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From the Geriatric Department, Södersjukhuset, Stockholm, Sweden

Inhibition of Thrombocytopenia and 5-Hydroxytryptamine Release in Anaphylactic Shock by Heparin

By

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Abstract

JOHANSSON, S.-A. *Inhibition of thrombocytopenia and 5-hydroxytryptamine release in anaphylactic shock by heparin.* Acta physiol. scand. 1960. *50.* 95—104. — After anaphylactic shock increased amounts of 5-HT in plasma, and lung tissues, a decrease in the circulating number of platelets and increase in the urinary excretion of 5-hydroxyindole acetic acid (5-HIAA) were found but no alteration in the amounts of 5-HT in the intestines. Pretreatment of the animals with heparin prevented these effects and also the release of 5-HT from cells in sensitized blood after addition of a specific antigen to citrate blood in vitro. Heparin also inhibits the ability of platelets to adsorb 5-HT in vitro. Dialysis experiments, coagulation time measurements and intravenous injections of 5-HT on heparin treated animals indicate that heparin has no ability to bind 5-HT in blood. It is suggested that heparin, being a strongly acid polyelectrolyte, acts on the platelets and their ability to absorb and release 5-HT and prevents the onset of a transient intravascular coagulation during anaphylaxis.

The anaphylactic phenomenon was first described by PORTIER and RICHET (1902) in a dog which had received sub-lethal doses of an extract of sea-actinia. ARTHUS in 1903 showed that the phenomenon of Richet occurred in the rabbit after repeated injections of nontoxic materials, such as egg albumin and horse serum. Anaphylaxis was defined by DRAGSTEDT (1941) as "an auto-intoxication

by physiologically active substances normally resident in various tissue cells and liberated therefrom by some change in cellular permeability brought about by antigen-antibody reaction".

The main characteristics of anaphylactic shock in most animal species are contraction of smooth muscle, oedema formation and a fall in blood pressure. Such symptoms can be produced by biologically active substances such as histamine and 5-hydroxytryptamine (5-HT, serotonin), the release of which accompany anaphylactic reactions in rabbits (DALE and LAIDLAW 1910, FISCHER and LECOMTE 1956). These two amines occur in large amounts in the rabbit blood platelets (HUMPHREY and JAQUES 1954). Anaphylactic shock is known to lead to a decrease in the number of circulating platelets (ACHARD and AYNAND 1909). A correlation between the severity of the shock and the thrombocytopenia in rabbits has been demonstrated by KOPELOFF and KOPELOFF (1941). By antigen-antibody reactions in vitro histamine and 5-HT are liberated from rabbit blood platelets suspended in plasma. (HUMPHREY and JAQUES 1955).

In a preliminary report it has been shown that the release of 5-HT during anaphylaxis in rabbits is prevented, if the animals receive large doses of heparin 20 min before the injection of the anaphylactic dose (JOHANSSON 1960). Heparin also prevents the local Schwartzman reaction (EICHBAUM 1957). The object of this investigation is to study the protective action of heparin in anaphylactic shock and the interaction between heparin and 5-HT.

Experimental

Rabbit experiments

Rabbits of both sexes weighing 2.5—3.0 kg were used in these experiments. The rabbits were sensitized with 1 ml horse serum i. p. daily during 6 days. Five weeks later when the titre of complement fixation antibody was high, the rabbits were given the anaphylactic dose (1 ml of horse serum diluted 1:50 in 0.9 per cent saline i. v.) Twenty minutes before the anaphylactic dose 5 rabbits had been given i. v. 100 mg of heparin Vitrum, containing 108 i. u. of heparin per mg. Ten sensitized rabbits, which had not received heparin served as a control group. The control group was given 1 ml of 0.9 per cent saline instead of heparin.

Blood samples were drawn by heart puncture or through a polyethylene catheter in the carotid artery at one hour before and at 1, 2, 3, 5, 10, 30 and 60 min after the injection of the anaphylactic dose. In most of the animals blood samples were also drawn by heart puncture or through a plastic tube in the carotid artery at 3, 6, 12, and 24 hours after the anaphylactic dose. Silicon treated vessels were used throughout. Nine volumes of blood were collected into centrifuge tubes containing 1 volume of 3.8 per cent trisodium citrate ($Na_3C_6H_5O_7$, 2 H_2O). International Co. lusteroid centrifuge tubes were used.

Platelet rich plasma was obtained by centrifuging citrated blood at 4° C at 185 *g* for 5 min. immediately after collection.

Platelet deficient plasma was prepared by centrifuging platelet rich plasma at 16,000 *g* for 30 min in plastic tubes at 4° C and then by pipetting off the supernatant plasma with-

out disturbing the layer of platelets. The platelets were then washed two times in saline. Platelet counts were made by the method of Kristenson.

Five animals were killed 1 hour after they had received the anaphylactic dose. Intestines and lungs were homogenized with 0.1 n HCl and their content of 5-HT was determined after extraction into n-butanol.

Coagulation time measurements were made according to HEDENIUS (1936).

Blood from sensitized rabbits was incubated at 37° C for 30 min with a specific antigen (horse serum diluted with one volume of saline solution. The tubes were swirled every 5 min. After centrifugation the diluted plasma was analyzed for 5-HT.

Experiments on human blood

Human platelets prepared as described earlier (NILSSON *et al.* 1957) were incubated in vitro at 37° C with excess 5-HT (2 μg/ml) together with or without heparin. At intervals their ability to absorb 5-HT was determined. The platelets were then washed three times in 0.9 per cent saline before the determinations. Platelet counts were made before and after the incubation.

Dialysis experiments

Dialysis tests were carried out to examine under which conditions 5-HT could combine with heparin. Ten ml samples of 5-HT and 10—50 mg of heparin in the buffer solution used). Dialysis was performed against 90 ml of outer liquid. The solutions used were 0.1 M sodium acetate — acetic acid (pH = 3.1) water, 0.9 per cent saline, 0.1 M phosphate buffer (pH = 7.4) (Ionic strength = 0.2) plasma and the carbonate buffer (pH = 10) described by UDENFRIEND, WEISSBACH and CLARK (1955).

Chemical analyses

The 5-HT concentrations in whole blood, platelet rich plasma, platelets and tissues were determined spectrophotofluorometrically after extraction into purified n-butanol according to UDENFRIEND, WEISSBACH and CLARK (1955, 1957). The 5-hydroxyindole acetic acid (5-HIAA) analyses in urine were carried out spectrophotometrically on 24 hours samples according to UDENFRIEND *et al.*, (1955) and MACFARLANE *et al.* (1956). Standards yields were determined and blanks were run with each series of determinations.

Results

Rabbits

The 5-HT content in whole blood and plasma at intervals after the anaphylactic dose is shown in Fig. 1 and 2. There was a marked decrease in the amount of 5-HT in blood after anaphylactic shock. There also was a prolonged diminution in the platelet count (Fig. 3). In plasma however, an increase in the 5-HT content occurred during the first 5 min after the anaphylactic dose. The platelet count regained normal levels within 2—3 hours, but the content of 5-HT in whole blood remained below normal levels for about 24 hours.

In those animals, which had been pretreated with heparin, the anaphylactic dose was accompanied by comparatively small changes in plasma 5-HT (Fig. 2). There only occurred a slight decrease in the 5-HT content of whole blood (Fig. 1) and no diminution in the number of circulating platelets was found (Fig. 3).

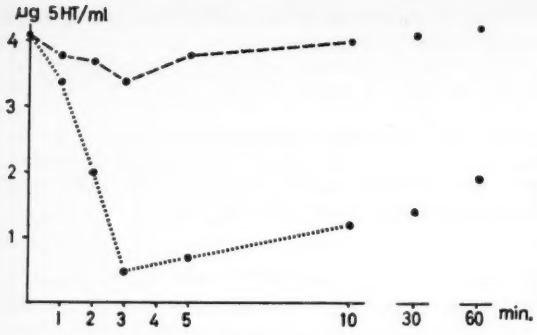

Fig. 1. The amount of 5-HT in whole blood at intervals after the anaphylactic dose.
Untreated animals .....
Heparin treated animals — — — —.

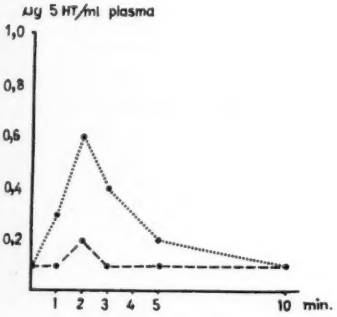

Fig. 2. The amount of 5-HT in plasma at intervals after the anaphylactic dose.
Untreated animals .....
Heparin treated animals - - - - - -.

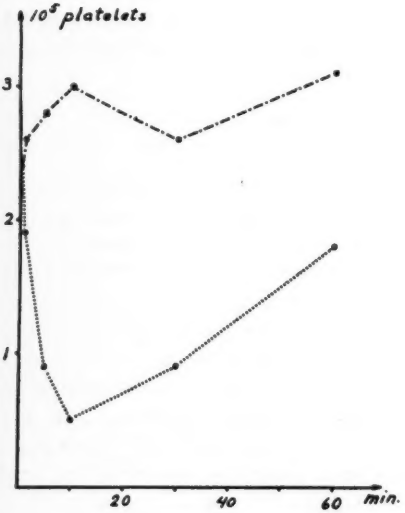

Fig. 3. Platelet counts during anaphylaxis.
Untreated animals .....
Heparin treated animals - - - - - - -.

Table I. Excretion of 5-HIAA mg per 8 hours during the first period after the anaphylactic shock of untreated and heparin treated rabbits compared to sensitized rabbits

	Sensitized	After anaphylactic shock	Heparin prophylactically before the anaphylactic shock
Number of animals	10	5	5
5-HIAA	1.0 ± 0.2	2.1 ± 0.3	1.0 ± 0.2

Table II. Amount of 5-HT in rabbit whole blood intestines and lungs 1 hour after the anaphylactic shock as compared to control values from sensitized rabbits. (Range used where the analyses differed by more than 1 μg/ml or g)

5-HT (μg/ml or g wet tissue)

	Sensitized	One hour after the anaphylactic dose	
		Untreated	Heparin treated
Number of animals	5	5	5
Intestine	8—15	7—13	7—12
Whole blood	4.0	1.9	4.2
Lung	2.6±0.3	3.5±0.3	2.5±0.2
	2.1—3.4	3.0—4.2	2.3—3.2

Table III. Amount of 5-HT in intestines and lungs 10 min after the anaphylactic shock to control values from sensitized rabbits. (Range used where the analyses differed by more than 1 μg/g wet tissue)

5-HT (μg/g wet tissue)

	Sensitized	10 minutes after the anaphylactic dose	
		Untreated	Heparin treated
Number of animals	5	5	5
Intestine	8—15	7—14	8—16
Lung	2.5—4.0	8.2—12.0	2.3—3.8

There also occurred an increase in the urinary excretion of the 5-HT metabolite — 5-HIAA — after anaphylactic shock in rabbits (Table I). In those animals which previously had been given heparin prophylactically no increase in the 5-HIAA excretion was found.

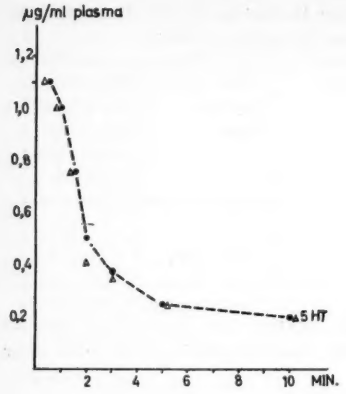

Fig. 4. Plasma content of 5-HT following intravenous injections of the amine into three heparin treated animals and three untreated control animals. (controls △)

The amount of 5-HT in intestines were high and because of the wide normal variation a release of the amine from the intestines would be difficult to detect. Analyses from 5 animals after anaphylactic shock gave values within the normal range (Table II). In the lungs, however, a large increase in the measurable amounts of 5-HT was found within 5 min after the anaphylactic shock. A small elevation of the 5-HT level in the lung was still observed one hour after the anaphylaxis. In the animals pretreated with heparin, no increase in the lung content of 5-HT was detected (Table III).

Fig. 4 shows that intravenous injections of 200 mg 5-HT creatinine sulfate to 3 rabbits, (which had been given 100 mg of heparin intravenously 30 min before the injection of the amine) rapidly disappeared from plasma.

5-HT is liberated into plasma after addition of a specific antingen to whole blood. The 5-HT values in the experimental plasmas were several times higher than the values for the control plasmas. If 10 mg of heparin per ml was added to the diluted whole blood 30 min before the specific antigen, no increase in the experimental plasma content of 5-HT was found (Table IV).

Table IV. Release of 5-HT from rabbit blood cells when a specific antigen is added to whole blood in vitro

5-HT µg/ml

	Number of rabbits	1:50 horse serum	0.9 % saline
Normal controls	5	0.2	0.1
Sensitized to horse serum Untreated	5	1.7	0.3
Treated with heparin	5	0.4	0.3

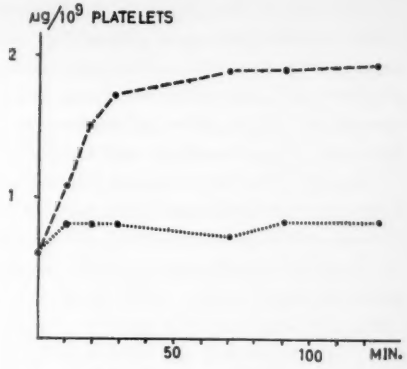

Fig. 5. The amount of 5-HT in human platelets after incubation with excess of 5-HT with and without heparin.
Heparin added to the incubation . . . . . .
(Each point represents the mean of 5 experiments.)

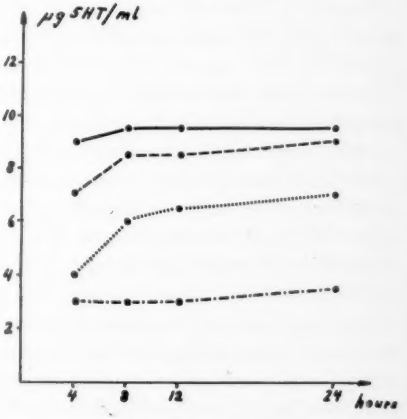

Fig. 6. Dialysis of 5-HT against heparin in water —.—.—.—.,
0.1 M sodium acetate . . . . . .,
0.9 % saline — — — — — — —,
5-HT in water without heparin ———.

Human blood

In vitro studies of human platelets showed that they were not saturated with 5-HT. Incubation at 37° C with excess of 5-HT greatly enhanced their content of 5-HT. If 10 mg heparin per ml solution was added the platelets lost their ability to absorb 5-HT (Fig. 5).

Dialysis

Dialysis of 5-HT solutions against heparin showed that in the acid aqueous solution and water, heparin is able to combine with 5-HT (Fig. 6). In plasma, a phosphate buffer of pH = 7.4 (ionic strength = 0.2), 0.9 per cent saline or a carbonate buffer of pH = 10, however, heparin had no ability to bind 5-HT. Heparin thus seemed to have no ability to combine with 5-HT in blood. 5-HT further had no effect on the coagulation time of heparinized rabbit blood (1 mg of heparin per ml blood) in vitro or in vivo in doses up to 500 5-HT μg per ml (in vitro).

Discussion

Sensitized pigeons and guinea pigs are protected from anaphylactic shock if heparin is given intravenously 20 min before the anaphylactic dose (WILLIAMS and VAN DER CARR 1927, KEYS and STRAUSSER 1927). A correlation between the severity of the symptoms in anaphylactic shock and the thrombocytopenia has been shown by KOPELOFF and KOPELOFF (1941).

The present study indicates that heparin prevents the decrease in the circulating number of platelets with ensuing decrease of 5-HT in whole blood, two phenomena which accompany the anaphylactic shock.

The increased amount of 5-HT in plasma and lung tissues accompanied by larger amounts of the 5-HT metabolite — the 5-HIAA — in urine probably originates from 5-HT in platelets, which are desintegrated at antigen — antibody reactions.

Sensitized rabbits pretreated with 0.1 mg/kg of reserpine, however, had no elevated plasma level of 5-HT, indicating that the major part of the rise in plasma of 5-HT during anaphylaxis is secondary to a release from platelets (WAALKES *et al.* 1957). These authors further found that the 5-HT content in the intestinal tract from 4 animals 24 hours after anaphylaxis were within the normal range. This study concur with these results.

No increase of 5-HIAA was observed in those rabbits which had received large doses of heparin prophylactically. As to the mechanism of the protective action of heparin in anaphylactic shock nothing is known. No effect of 5-HT on coagulation time of heparinized blood was observed when doses up to 500 μg 5-HT per ml was added in vitro. Nor can large doses of heparin prevent the rapid disappearance from blood of intravenously injected 5-HT. The present study indicates that heparin has no or very little ability to combine with 5-HT at a pH and a salt concentration corresponding to that in blood. Heparin thus probably does not act in blood by binding released 5-HT.

The concentration of 5-HT in rabbit platelets is several thousand times that in plasma. The rate with which indole compounds pass into the platelets has been studied by BRODIE and HOGBEN (1957). They suggest that 5-HT is transported into the platelets by a special mechanism against a steep concentration gradient.

Thrombocytopenic blood can be kept fluid for hours by addition of a small amount of heparin (ALLEN *et al.* 1947). Whether the platelets absorb heparin on their surface or not is not known. This study shows that the platelets lose their ability to absorb 5-HT, if heparin is added to the plasma. Heparin probably acts on the platelets and their ability to absorb and liberate 5-HT because it is a strongly acid polyelectrolyte, which may be able to inhibit the transport mechanism probably needed for those processes. Of interest would be to study whether a dextran sulfate of low molecular weight, chondroitin sulfuric acid and similar compounds behave similarly to heparin in preventing the decrease in platelet count and the release of 5-HT in anaphylaxis.

It is interesting to note that allergic reactions often are accompanied by a decrease in the number of circulating platelets. Hypersensitive patients have been given subcutaneous doses of a specific allergen, large enough to produce a distinct decrease in platelet count and local or general symptoms. These events were prevented by i. v. injections of heparin 30 min. prior to the injection of allergen (JOHANSSON, LUNDBERG and SJÖBERG 1960). It has however not been established whether 5-HT contributes to the anaphylactic reaction in man.

Many of the effects produced by 5-HT resemble the manifestations of hypersensitive reactions (UDENFRIEND *et al.* 1957). Further, patients with malignant carcinoid often show symptoms resembling those of an allergic process. In mouse 5-HT has been implicated as the major toxic agent in anaphylaxis (studied in vitro, FINK 1956).

The released amount of 5-HT in rabbit blood during anaphylaxis are too small to cause death injected intravenously to rabbits. The 5-HT probably is not the major toxic substance in anaphylaxis but many of the contributing symptoms which appear might be produced by 5-HT.

Heparin shows a diversity of biological activity. Heparin has been shown to inhibit enzyme activity and, like other polyanionic colloids, form insoluble salt with proteins. (JORPES 1946). The anti-thrombin effect of heparin in coagulation is well known. A possible role of heparin in anaphylaxis would therefore be to prevent the onset of an intravascular coagulation during anaphylaxis. Preliminary results have shown that most of the coagulation factors are consumed during anaphylaxis and that large amounts of an anti-thrombic substance, probably heparin, is released into rabbit plasma during anaphylaxis (BLOMBÄCK, JOHANSSON, SJÖBERG 1960). The incoagulability of dog's blood in anaphylaxis is caused by heparin (JAQUES and WATERS 1941). The appearing symptoms and death in anaphylaxis might then be due to a transient intravascular coagulation with embolies and thromboses which block the circulation in the vessels.

Heparin thus may have a protective action in anaphylaxis by its direct action on platelets and its influence on different humoral coagulation factors.

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Inhibitory Effects of Hydrochloric Acid in the Duodenum on Gastrin-Stimulated Gastric Secretion in Heidenhain Pouch Dogs

By

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Abstract

ANDERSSON, S. *Inhibitory effects of hydrochloric acid in the duodenum on gastrin-stimulated gastric secretion in Heidenhain pouch dogs.* Acta physiol. scand. 1960. *50*. 105—112. — In Heidenhain pouch dogs with duodenum excluded from the normal gastrointestinal passage, gastric secretion was stimulated by continuous intravenous infusion of a gastrin preparation from hog's antral mucosa. On instillation of hydrochloric acid into the duodenum the secretion was decisively inhibited. This finding was taken to indicate that the activated pH-sensitive inhibitory mechanism in the duodenum exerts its action directly upon the parietal cells.

Previous investigations (ANDERSSON 1960 a, b, c) have amply demonstrated the inhibitory effect upon the gastric secretion of HCl instillation into the duodenum. In those studies reduction of the intraduodenal pH depressed the secretion from both Pavlov and Heidenhain pouches. Since the inhibition is accordingly independent of vagal innervation, it may be assumed to act either via liberation of a secretion-inhibiting factor from the duodenal mucosa or via secretion-inhibiting reflexes in sympathetic nerves. Theoretically, each of these mechanisms might act either by direct inhibition at parietal cell level or by blocking the release of gastrin from the antral mucosa. Earlier studies have not completely ruled out either of these alternatives.

Gastrin is a secretagogue which occurs physiologically and can be extracted from the antral mucosa (KOMAROV 1942, UVNÄS 1942, JORPES, JALLING and

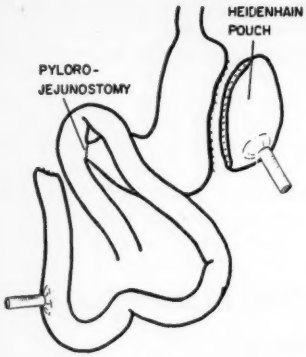

Fig. 1. Operative preparation of the dogs: Heidenhain pouch; pylorojejunostomy and cannula in the duodenum.

MUTT 1952). By studying the susceptibility of gastrin-stimulated secretion to inhibition via the duodenum, the writer has sought to clarify the mode of action of the duodenal mechanism.

Methods

Four adult mongrel dogs weighing 15—20 kg were prepared in two stages. At the initial operations Heidenhain pouches were established. About three weeks later, when the animals had recovered from this operation, they were subjected to the following surgical measures: The pylorus was divided and the duodenum closed. A new gastrointestinal passage was created by anastomosing the first loop of the jejunum to the divided pylorus. A cannula was inserted into the duodenum approximately 10 cm distal to its upper invaginated end. The preparation is illustrated in Fig. 1. Before the initiation of experiments the animals were allowed at least three weeks to recover. For details of the operative method and postoperative management, the reader should refer to ANDERSSON, ELWIN and UVNÄS (1958) and ANDERSSON (1960 b).

Each experiment was started in the forenoon after the animals had fasted for 18—24 hours. The basal secretion was recorded for at least one hour before stimulation of the secretion began. The secretagogue used in these experiments was gastrin (for mode of preparation, see below). The gastrin preparation was dissolved in sterile physiological saline in concentrations of 0.5—1.5 mg/ml. With the aid of an automatic infusion pump, it was continuously administered intravenously over 4—6 hours, a constant secretory level generally being reached after about one hour. The doses administered varied between 8 and 19 μg/kg/min, depending upon the secretory responses of the Heidenhain pouches to gastrin.

The gastric secretory output was collected in 15-min portions and the amounts of free HCl and total acid were determined by titration of each portion against N/100 NaOH, with Töpfer's reagent and phenolphthalein as indicators.

The effects of intraduodenal infusions of hydrochloric acid on the secretion were studied both during the initial phase of the secretory response and during its later stages when the level was constant. The acid infusions proceeded for 1—$1^1/_2$ hours, the amounts of acid being so adjusted as to reduce the intraduodenal pH from 7—8 before the infusion to approximately pH 2 during the infusion period. For this purpose the amounts required were approximately 100 ml N/5 HCl per hour.

Table I. Hourly secretory responses to gastrin in Heidenhain pouch dogs with and without concomitant instillation of hydrochloric acid into the duodenum

A. Dog 129. Gastrin dose 8.1—8.9 μg/kg/min.

	Exp. no.	Secretion (mEq total acid)		
		Control period (one hour)	Response to gastrin	
			1st hr	2nd hr
Controls	1	0.14	1.00	1.18
	2	0.06	0.53	0.81
	3	0.11	1.15	1.64
	Mean	0.10	0.89	1.21
N/5 HCl in duodenum during the 1st hour	1	0.05	**0.34**	1.06
	2	0.06	**0.28**	0.88
	3	0.06	**0.20**	1.13
	Mean	0.06	**0.27**	1.02
In percent of mean for controls.....		—	**30**	84

Further details of the infusion technique and method for determination of the intraduodenal pH will be found in an earlier paper (ANDERSSON 1960 b).

Preparation of Gastrin

Gastrin was prepared from antral mucosa of hog, in principle by the following method, modified after JORPES *et al.* (1952):

The antral portion of the hog stomach was separated and freed of mucus by washing. The mucous membrane was dissected off and boiled in neutral water for ten minutes. Mucous membranes thus obtained from 600 hog stomachs were minced and subjected to extraction with N/10 HCl in 95 per cent methyl alcohol. After filtration of the suspension, the filtrate pH was raised first to 5, then to 5.5. Precipitates occurred at each of these pH levels and were filtered off and discarded. The solution was neutralized (pH 7) and the formed precipitate collected and dissolved in acid methyl alcohol, after which ether was added. The precipitate was filtered off and washed in ether. The filter cake was dissolved in distilled water and the pH of the solution adjusted to 7. The precipitate was collected by centrifugation, dissolved in the minimal amount of distilled water at pH 5 and freeze dried. The dry preparation weighed approximately 1 g.

The preparation, when tested for its histamine content on guinea-pig ileum, was found to contain less than 0.1 μg histamine per milligram. The amount of histamine which the administered doses might have contained was estimated to be about 100 times too small to induce secretion from the Heidenhain pouches.

Table I.

B. Dog 143. Gastrin dose 8.7—9.6 μg/kg/min.

	Exp. no.	Secretion (mEq total acid)					
		Control period (one hour)	Response to gastrin				
			1st hr	2nd hr		3rd hr	
				1/2 hr	1/2 hr	1/2 hr	1/2 hr
Controls	1	0.04	0.69	0.35	0.46	0.45	—
	2	0.02	0.44	0.39	0.44	0.45	—
	3	0.02	0.63	0.46	0.41	0.43	—
	Mean	0.03	0.59	0.40	0.44	0.44	—
N/5 HCl in duodenum during the 1st and half of the 2nd hour	1	0.08	**0.19**	**0.13**	0.22	0.37	—
	2	0.06	**0.21**	**0.18**	0.28	0.44	—
	Mean	0.07	**0.20**	**0.16**	0.25	0.41	—
In percent of mean for controls		—	**34**	**40**	57	93	—

Results

The effect of HCl infusion into the duodenum upon gastrin-stimulated secretion was studied in 14 experiments on four dogs. Experiments of two types were conducted: In series A, acid instillation into the duodenum and stimulation of the secretion began simultaneously; in series B the acid infusion was not begun until the secretion had reached a constant level.

Seri s A (Dogs 129 and 143)

The secretory responses of the Heidenhain pouches to continuous intravenous infusion of gastrin were determined and the results are detailed in Table I. The effect of a low intraduodenal pH on these responses was studied in five experiments. On reduction of the pH to about 2 by continuous infusion of approximately 100 ml N/5 HCl into the duodenum, the secretion was inhibited by an average of 60—70 per cent (Table I).

Series B (Dogs 132, 136 and 143)

The secretory responses to gastrin usually reached after one hour a relatively constant level, which could then be maintained for 3—5 hours. This made it possible to record in any single experiment both the ordinary secretory level and the effect thereon of intraduodenal instillation of acid. The secretory response was studied for 2—2$^1/_2$ hours, whereafter HCl was instilled for 1 hour. Following this instillation the secretion was recorded for a further 1—1$^1/_2$ hours.

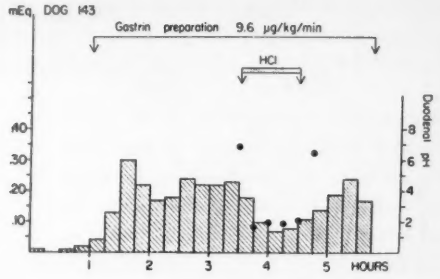

Fig. 2. Inhibition of gastrin-induced gastric secretion in a Heidenhain pouch dog by instillation of 100 ml N/5 HCl per hour into the duodenum. (• : intraduodenal pH)

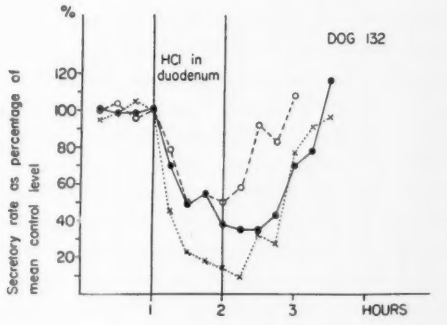

Fig. 3. Inhibition of gastrin-induced gastric secretion from Heidenhain pouches by instillation of hydrochloric acid into the duodenum (intraduodenal pH about 2). Each curve represents a separate experiment. The 100 percent level is the mean 15-min gastric secretion (control level) during the hour immediately preceding the infusion of acid.
A. Gastrin dose 8.3—8.6 µg/kg/min. Mean secretory control level varied between 0.22 and 0.35 mEq acid per 15 min.

A typical experiment is illustrated in Fig. 2. Here the secretion was stimulated with gastrin 9.6 µg/kg/min, which produced a response of about 0.20 mEq acid per 15 min. Instillation of about 100 ml N/5 HCl per hour into the duodenum reduced the intraduodenal pH to approximately 2, whereby the secretion was progressively inhibited. The maximum secretory inhibition amounted in this instance to about 70 per cent. Consistent results were obtained in nine such experiments on the three animals. These results are condensed in Fig. 3 a, b and c. The mean secretory output per 15 minutes during the hour immediately preceding the acid infusion is taken as 100 per cent (control level). The recorded inhibitory effects are expressed in percent of the control level. It will be seen that the depression was more or less equally pronounced in the different experiments on the three animals. In no case was total suppression produced.

No inhibition was observed in experiments wherein, due to insufficient acid administration, the intraduodenal pH did not fall to about 2.

Instillation of physiological saline into the duodenum had no inhibitory effect upon the secretion.

The results did not differ in principle when the secretion was expressed in milliequivalents of free HCl instead of milliequivalents of total acid.

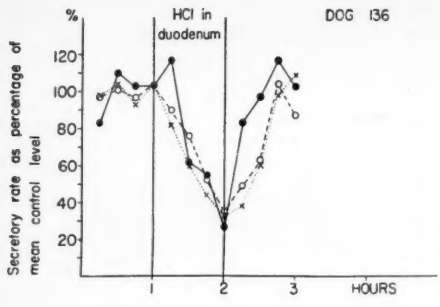

Fig. 3. B. Gastrin dose 18.5—18.8 μg/kg/min. Mean secretory control level varied between 0.15 and 0.29 mEq acid per 15 min.

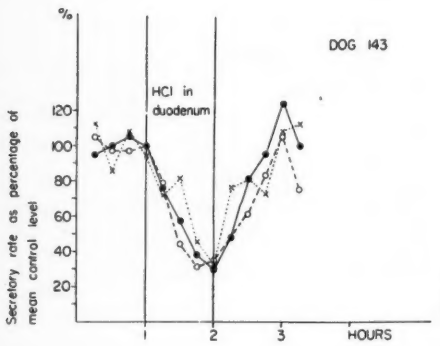

Fig. 3. C. Gastrin dose 9.4—9.6 μg/kg/min. Mean secretory control level varied between 0.21 and 0.23 mEq acid per 15 min.

Discussion

It was shown in previous investigations that a low intraduodenal pH has an inhibitory influence on the fasting and postprandial secretion from both Pavlov and Heidenhain pouches (ANDERSSON 1960 a, b) as well as on insulin-stimulated secretion from Pavlov pouches (ANDERSSON 1960 c). In none of these investigations was it possible to localize the site of action of the inhibitory mechanism. Two alternatives are conceivable: either the inhibition occurs at parietal cell level, or the liberation of gastrin from the antral mucosa is blocked.

It was for this reason that the present study was concerned with the effect of a low intraduodenal pH on secretion stimulated by gastrin. The latter stimulus is preferable not only because it may be regarded as a physiologic secretagogue but because, on continuous intravenous infusion, it causes a relatively constant secretion, inhibition of which can easily be recorded.

The results show that gastric acid secretion induced by gastrin from Heidenhain pouches is substantially inhibited by lowering the intraduodenal pH to about 2. This observation strongly suggests that the activated inhibitory mechanism has its site of action at the parietal cell level.

GREGORY and TRACY (1959) reported that fat in the duodenum reduced secretion which had been stimulated by irrigation of an isolated antrum pouch with meat extracts or acetylcholine solution. Histamine-induced secretion, on the other hand, was not inhibited. In their opinion "the action of fat in the duodenum is to inhibit stimulation of gastric acid secretion by the antral mechanism". They had no gastrin preparation available and were therefore unable to determine if the mechanism acted by reducing the release of gastrin or by blocking its action upon the parietal cells.

JONES and HARKINS (1959) consider that acid in the duodenum exerts a hormonally induced inhibition of gastric acid secretion. They stimulated the secretion with a test meal consisting of 100 ml 10 per cent ethyl alcohol. Instillation of acid into the duodenum reduced that secretion and the inhibition was more pronounced in Heidenhain than in Pavlov pouch dogs. Their investigation is, however, inconclusive inasmuch as they recorded only the acidity of the gastric juice. The importance of quantitative determinations in studies of gastric secretion is evident from the following considerations: In general the secretion from Heidenhain pouches is meagre, the amounts of acid being small by comparison with the secretory responses from Pavlov pouches. When an inhibitor acts upon the secretion, low secretory volumes are associated with a greater reduction of acidity since the admixture of mucus is comparatively greater than that in corresponding inhibition of profuse secretory responses. This may account for the fact that JONES and HARKINS observed the best inhibitory effects on secretion from Heidenhain pouches.

It is possible that fat and acid in the duodenum have, in principle, the same inhibitory mechanism; namely, the liberation from the duodenal mucosa of a secretion-inhibiting factor which, by the humoral route, *interferes directly* with the function of the parietal cells.

The significance of the sympathetic innervation of the stomach in its secretory functions is, as pointed out earlier (ANDERSSON 1960 a, b), unknown. An ordinary Heidenhain pouch doubtless has a relatively large portion of its sympathetic innervation intact.

Until the influence of a low intraduodenal pH on different types of secretion from totally denervated gastric pouches has been studied, the question as to whether the pH-sensitive inhibitory mechanism in the duodenum is of humoral or of nervous nature cannot be definitely answered.

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Acta physiol. scand. 1960. 50. 113—118

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"Bound" and "Free" Catecholamines in the Brain

By

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Abstract

Bertler, Å., N.-Å. Hillarp and E. Rosengren. *"Bound" and "free" catecholamines in the brain.* Acta physiol. scand. 1960. *50.* 113—118. — The major portion of the noradrenaline in the rabbit hypothalamus was recovered in the particulate fraction of homogenates in 0.3 M sucrose. In contrast to this, the dopamine in the caudate nuclei of rabbit and cat was to the largest part found in the high-speed supernatant. The amine distribution did not seem to be materially influenced by the use of different homogenization media. Neither were any obvious changes in the distribution seen after administration of iproniazid or reserpine. The dopamine formed in the hypothalamus after an injection of L-dopa was found to be "particle-bound" to a rather large extent.

There is good evidence that the catechol amines in the adrenal medulla and adrenergic nerves are stored in cytoplasmic granules in a bound state (*cf.* Carlsson and Hillarp 1958, Hillarp 1959, Euler 1958, Schümann 1958); but little is known about the way in which the amines in the brain are stored. Walaszek and Abood (1957) and Weil-Malherbe and Bone (1957) have shown that half or more of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) is recovered in the mitochondrial fraction when brain homogenates are examined by differential centrifugation. It may thus be that these amines — at least partly — are stored in a similar way as those in the adrenal medulla. Such a view is also supported by the effect of reserpine generally to deplete the body stores of catechol amines (Carlsson *et al.* 1957).

Table I. Content and intracellular distribution of noradrenaline and dopamine in the brain of rabbit and cat.

Rabbit 6 was killed with a large intravenous dose of potassium cyanide. Rabbit 7 was bled to death in deep Nembutal anesthesia. The cats were bled to death in deep nitrogen monoxide anesthesia.

The amine content in the high-speed sediment is expressed in per cent of the amines in the low-speed supernatant.

		Treatment of Animal	Homogenization Medium	Hypothalamus: NA		Caudate Nuclei: DA	
				Total μg	High-speed sediment Per cent	Total μg	High-speed sediment Per cent
Rabbit	1	None	0.3 M sucrose	0.68	77		
	2	»	»	0.38	68		
	3	»	»			1.4	11
	4	»	»			1.2	39
	5	»	»	2.1	43	1.8	42
	6	Killed with KCN	»	0.43	67	0.80	39
	7	Killed in Nembutal anesthesia	»	0.40	67	1.5	34
	8	None	0.3 M sucrose with 0.002 M $CaCl_2$	1.1	57	2.3	35
	9	»	0.3 M sucrose with 0.03 M K-phosphate pH 7.4	0.61	72	2.3	29
	10	»	0.3 M sucrose with 10 % bovine serum albumin	0.47	65	1.4	24
	11	»	0.3 M sucrose with 5 % dextran	1.2	34	2.0	18
	12	»	0.2 M KCl with 0.002 M $CaCl_2$	1.1	75	2.4	36
	13	»	Krebs-Ringer-phosphate	0.58	71	2.2	68
	14	»	»	0.55	56	2.0	38
	15	»	»	0.72	73	1.7	60
Cat	1	Killed in N_2O anesthesia	0.4 M sucrose with 0.01 M Versen pH 7.4			4.7	14
	2	»	0.5 M sucrose			5.6	27
	3	»	0.2 M KCl with 0.002 M $CaCl_2$			4.7	25
	4	»	»			4.6	20

Table II. Content and intracellular distribution of noradrenaline and dopamine in the rabbit's brain.
Homogenization was performed in 0.3 M sucrose. All substances were given intravenously. The amine content in the high-speed sediment is expressed in per cent of the amines in the low-speed supernatant.

Rabbit	Treatment of Animal	Hypothalamus				Caudate Nuclei			
		Total µg		High-speed sediment Per cent		Total µg		High-speed sediment Per cent	
		NA	DA	NA	DA	NA	DA	NA	DA
16	25 mg L-dopa/kg 15 min	0.69	0.99	74	73		2.5		45
17	»	0.52	0.95	70	51		2.1		35
18	»		0.65		45		2.6		30
19	»	0.58	0.57	67	51		1.7		31
20	»	0.65		74			2.0		2
21	100 mg »	1.2	1.8	56	39		3.7		30
22	1 mg reserpine/kg 10 min	0.44		71			0.88		42
23	»	0.68		77			0.77		30
24	»	0.88		70			2.2		42
25	100 mg iproniazid/kg 6 hr	0.53		74			1.7		42
26	»	0.54		74			2.9		43
27	100 mg iproniazid/kg 5 hr 1 mg reserpine/kg 1 hr ..	0.49		70			2.2		61
28	»	0.59		63			1.0		36
29	Homogenization in 0 3 M sucrose with 1.6 µg dopamine	0.68	1.3	77	20				
30	»	0.38	1.5	68	20				
31	As in 29 but with 2 µg noradrenaline.....					1.7	1.4	24	11
32	»					1.8	1.2	13	39
33—35	Homogenization of cerebellar cortex (400 mg) in 0.3 M sucrose with about 1.6 µg dopamine.....		1.4		10				
			0.9		10				
			1.1		10				

Besides NA and 5-HT, dopamine (DA) is also present in the brain (CARLSSON *et al.* 1958). Practically the whole amount of this amine occurs in the corpus striatum (BERTLER and ROSENGREN 1959 a). Preliminary experiments on cats showed unexpectedly that only a minor portion of DA in the caudate nucleus — in contrast to NA in the hypothalamus — was recovered in the particulate fraction of homogenates. This prompted a more extensive investigation of the problem of "bound" and "free" catechol amines in the brain tissue.

Material and Methods

In a few preliminary experiments cats were used, but in all the other rabbits (body wt usually about 2 kg). Unless otherwise stated, the animals were killed by a blow on the head and decapitation. The hypothalamus (wt: 300—500 mg) and caudate nuclei (wt of both: 225—425 mg) were rapidly dissected and chilled with ice. A rapid rather than a clean and quantitative dissection was aimed at. This is probably the explanation of the variability in the figures for total amine content (Table I and II).

The tissue was very gently homogenized with a loose-fitting plastic pestle (about 1,000 rpm) for about 20 sec in 7 ml of 0.3 M sucrose (or other media). To remove unbroken tissue, cells and nuclei, the homogenate was centrifuged at 800 × *g* for 6 min. The supernatant was then centrifuged at about 44,000 × *g* for 50—60 min. The final supernatant was carefully sucked off and treated with perchloric acid (final concentration 0.4 N) and the low- and high-speed sediments were extracted with 0.4 N perchloric acid. — All operations were performed at 0°.

The NA and DA content in the extracts was determined spectrophoto-fluorimetrically (Bertler, Carlsson and Rosengren 1958, Carlsson and Waldeck 1958).

The low-speed sediment usually contained less than 15 per cent of the total amine content of the homogenates. The degree of homogenization of the brain tissue was thus satisfactory.

Results

The larger part (usually 60 to 70 per cent) of the NA in the hypothalamus was recovered in the high-speed sediment when the tissue was homogenized in 0.3 M sucrose (Table I and II). In no instance, however, was more than 77 per cent found in the particulate fraction. Various additions to the sucrose solutions or the use of electrolyte media did not seem to improve the recovery of "bound" NA.

The distribution of DA in the homogenates of the caudate nuclei — although more variable — almost regularly showed quite a different picture. In practically all the experiments the high-speed supernatant contained the larger part of the amine, and in many of them "bound" DA represented less than 35 per cent. The DA distribution did not seem to vary with the way in which the animals were killed or the use of various additions to the homogenization media or of other media (see Table I).

The DA found in the high-speed supernatant might be bound to particles difficult to sediment. This does not seem likely, however. In the experiments where electrolytes were used in the homogenization media the final supernatant was completely clear showing that most of the submicroscopical particles had sedimented. Further, in an experiment with a sucrose homogenate centrifuged at about 150,000 × *g* for one hour the recovery of "bound" DA was not improved.

On the other hand, it might be argued that the recovery of NA and DA in the particulate fraction is only an artifact owing to an adsorption of the amines to cell particles, which are not storage structures in the living cell. Experiments 29 to 35 (Table II) show, in fact, that NA or DA added to the suspension medium may to some extent be adsorbed to cytoplasmic particles.

This phenomenon may thus give rise to serious errors, at least in the cases where less than half of the amines are recovered in the particulate fraction.

After an intravenous injection of a large dose of L-dopa, DA is rapidly formed and accumulates in the hypothalamus and the caudate nuclei (CARLSSON *et al.* 1958, BERTLER and ROSENGREN 1959 b). No certain change in the DA distribution in the caudate nuclei were found after dopa administration (exp. 16—21, Table II). The DA formed in the hypothalamus was, however, unexpectedly found to be "particle-bound" to a rather large extent even 15 min after the injection.

The administration of iproniazid or reserpine (Table II) did not seem to give any obvious changes in the NA and DA distribution.

Discussion

WEIL-MALHERBE and BONE (1957) and GREEN, SAWYER and ERICKSON (1959) found NA in the brain of rabbit and rat to be about equally distributed between the high-speed sediment and supernatant. In the present experiments somewhat higher recoveries of "bound" NA but considerably lower recoveries of "bound" DA usually were obtained. Using exactly the same technical procedures for fractionation of adrenal medulla of rat and rabbit it has regularly been found in our laboratories that 90 per cent or more of the catechol amines are recovered in the amine granules showing that a very small portion — if any — of the medullary amines occurs as free amines (*cf.* HILLARP 1960). There is thus an interesting difference between the adrenal medulla and brain tissue in this respect. It may be that this difference is of significance since the brain tissue is so easy to homogenize that the low recoveries probably cannot be ascribed to technical difficulties.

It is obviously at present impossible to decide in what way the brain amines are stored. The experiments clearly show that DA and NA may to some extent be unspecifically adsorbed to subcellular particles during the fractionation and that this phenomenon may give serious errors in the distribution figures. In spite of this, however, it seems probable that the amines — at least partly — are stored in cytoplasmic particles. These postulated storage granules seem to differ in some respects from the amine granules in the adrenal medulla since recent experiments by CHRUSCIEL (1960) suggest them to have different sedimentation characteristics.

The finding that the bulk of the DA in the caudate nuclei — in contrast to NA in the hypothalamus — was recovered in the "cytoplasmic sap" and that the distribution not seems to be materially influenced by the mode of killing of the animals or by the use of different homogenization media is interesting. However, the significance of this is entirely obscure. Far more extensive research is necessary before it is possible to decide whether the amines recovered in the final supernatant exist free in the living cell or whether they are released *post mortem* (*cf.* HILLARP 1960).

This work has been supported by grants from the Swedish Medical Research Council, the Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force, and from the Medical Faculty, Lund.

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From The Clinical Laboratory, The Royal School of Dentistry, Stockholm, Sweden

The Biological Half-Life of Bromide Ions in Human Blood

By

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Abstract

SÖREMARK, R. *The biological half-life of bromide ions in human blood.* Acta physiol. scand. 1960. *50*. 119—123. — The biological half-life of bromide ions in human blood was studied in 10 human subjects. The subjects were healthy students about 20 years old. The radioactive isotope, Br^{82}, was given orally in form of NH_4Br in water solution. Venous blood samples were drawn from the subjects twice a day. The biological half-life of bromide ions in the blood was found to be about 12 days. The bromide ratio red blood cells/serum was found to be about 0.45. This was calculated by using the mean from ten hematocrit values obtained from every person. No bromide was found to be incorporated in fat, extracted from plasma and hemolysed blood cells by ether. Neither was there found any bromide incorporated in blood proteins. This was determined by precipitation with trichloroacetic acid, tannic acid, and phosphotungstic acid and anion exchanger, and paper electrophoresis.

In a previous paper by SÖREMARK (1960) some findings have been reported on the distribution and kinetics of bromide ions in mice, rats, and rabbits studied by means of the two radioactive bromine isotopes Br^{80m} and Br^{82}. The biological half-life of the bromide ions in the blood of mice was found to be 1.5 days. The excretion of bromide which could be demonstrated was mainly renal. The radiobromide secreted by the gastric mucosa was rapidly reabsorbed in the intestine, and the radiobromide content of the feces was thus very small. It seems to be of importance to follow up these experimental investigations with a determination of the rate of elimination of bromide ions from the human blood.

Methods

Ten students, six men and four women, were used in this study. The subjects were selected on characteristics of good general health and they engaged in normal duties and enjoyed a normally varied diet. The age varied between 19 and 23 years, and the weight of the women between 55 and 64 kg and of the men between 62 and 78 kg. The height varied for the women between 163 and 175 cm and for the men between 172 and 184 cm.

To all the human subjects was given orally 10 μC of Br^{82} in the form of NH_4Br dissolved in 25 ml water. This corresponded to less than 0.05 mg ammonium bromide.

Blood samples of about 7 ml were drawn without stasis from the antecubital vein with heparinized syringes. The samples were taken twice a day, at 11.30 and 15.30. To avoid errors due to sedimentation of cells the samples were carefully mixed before 5.00 ml was taken for measurement of the radioactivity with the well-type scintillation detector. In order to determine the volume ratio between cells and plasma, blood samples were placed in calibrated glass tubes and centrifuged (VEALL and VETTER 1958). The individual hematocrit was thus measured for each person in the usual way each time blood samples were taken. The mean from the ten hematocrit values obtained from every person was then used when the radioactivity in the blood cells was calculated.

Fatty substances were extracted from the plasma and the hemolysed blood cells by ether for 1 hour. After centrifugation the supernatant was taken for measurement of radioactivity.

In order to determine if Br^{82} was incorporated in proteins of blood the following methods were used:

1) paper electrophoresis

2) precipitation with trichloroacetic acid (TCA), tannic acid, and phosphotungstic acid

3) anion exchange with Amberlite, IRA 400.

1) Paper electrophoresis of plasma samples was performed *ad modum* DETTKER and ANDURÉN (1954). The strips were dried and then cut into halves, one of which was stained with brom phenol blue for localization of the protein fractions. The other half was cut into 0.5 cm wide pieces for measurement of the radioactivity.

2) One ml 40 % TCA was added to the plasma and the hemolysed blood cell samples. The precipitates were washed 5 times with 5 % TCA. The washing solutions were added to the TCA soluble fraction. Tannic acid and phosphotungstic acid were added to the plasma and hemolysed blood cell samples until complete precipitates were obtained. After centrifugation the supernatant and the precipitate were assayed for radioactivity. The sediment was first washed 5 times in tannic acid and phosphotungstic acid respectively. The washing solutions were added to the soluble fractions.

3) The sample solutions of plasma and hemolysed blood cells were passed through the anion exchanger and the radioactivity measured in the solution which passed the column.

The biological half-life of bromide in human blood was calculated *ad modum* KAMEN (1957):

$$\frac{1}{Te\frac{1}{2}} = \frac{1}{Tb\frac{1}{2}} + \frac{1}{T\frac{1}{2}}$$

$Te\frac{1}{2}$ denotes the effective half-life, the time required for half of the Br^{82} ions to disappear from the body by radioactive decay and by normal biological processes. $Tb\frac{1}{2}$ denotes the biological half-life, the time required for half of the Br ions to be removed from the body by normal biological processes. $T\frac{1}{2}$ denotes the physical half-life of Br^{82}.

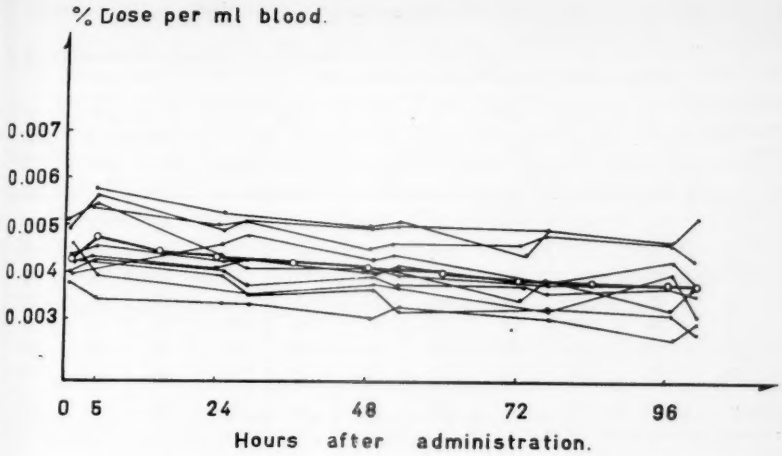

Fig. 1. The amount (expressed in per cent) of the given dose of bromide that remained in every ml of blood at various times after administration. The mean as well as the individual values are plotted.

Results

The concentration of Br^{82} in the blood at different times after the oral administration of NH_4Br^{82} solution is given in Fig. 1; the mean concentration as well as the individual values are plotted. It was observed that the radioactivity varied between 200 and 400 counts per minute per ml blood at five hours after Br^{82} was given.

No significant difference in the concentration, related to the time of the day at which the measurement was made, could be observed. The highest concentration was reached within 1—4 hours after the radioactive solution had been taken.

The individual concentration of radiobromide in blood cells was determined by measuring the Br^{82} concentration of the whole blood sample and of the sample of serum. The amount of Br^{82} ions transferred to the cells was calculated with the use of the hematocrit values. The results are given in Table I. In the human subjects studied it was found that of the whole amount of Br^{82} in the blood, between 26 and 34 % was transferred into the cells. The ratio red blood cells/serum of bromide ions was about 0.45.

The effective half-life of Br^{82} ions in human blood was found to be 32 hours and the biological half-life about 12 days as can be seen in Table II.

In no case were significant amounts of Br^{82} observed in fat or proteins.

Table I. Br^{82} in blood cells in per cent of the activity of whole blood after oral administration of NH_4Br^{82} to 10 human subjects

Time in hours after oral administr.	Subjects with individual haematocrit values									
	♀ 38% U. A.	♀ 41% B. J.	♀ 37% B. A.	♀ 44% K. C.	♂ 43% R. E.	♂ 43% B. L.	♂ 44% L. S.	♂ 44% Y. W.	♂ 43% G. E.	♂ 42% J. S.
	%	%	%	%	%	%	%	%	%	%
1	30	26	31	33	29	32	31	30	34	33
5	30	26	31	33	35	37	36	36	32	32
25	29	23	30	35	36	27	31	32	38	30
30	29	27	30	34	30	27	33	30	41	32
50	30	29	33	32	31	30	35	32	32	32
70	23	27	30	30	27	32	34	36	30	30
80	25	25	29	32	38	31	34	30	30	35
Mean	29	26	31	33	34	32	34	32	34	32

Discussion

Bromine belongs to those elements which are present in very small amounts in the body. CONWAY and FLOOD (1936) found an average value for human blood of 3.7 mg/l, however, with great individual variations. A ratio Br/Cl of roughly 1 : 1 000 was found in blood and urine. In drinking water they also found the same ratio. As the intake of bromide varies from one individual to another, a variation of the bromide level in blood might be expected.

When the radioactivities in red cells and plasma were measured, the hematocrit method was found to be accurate. For the same person the hematocrit varied less than $\pm$ 3 per cent. The ratio blood cells/serum of bromide ions was found by WEIR and HASTINGS (1939) to be 0.75, while GAMBLE *et al.* (1953) claimed that this ratio was 0.55. In the present investigation the corresponding figure was found to be 0.45.

Table II. Per cent Br^{82} remaining in the blood at various times after an oral dose of NH_4Br^{82}. Mean from 10 human subjects

4.5 h	91.2 $\pm$ 2.6 %
24 h	59.3 $\pm$ 2.5 %
29 h	52.1 $\pm$ 3.2 %
48 h	35.0 $\pm$ 1.6 %
52 h	31.9 $\pm$ 2.0 %
72 h	20.6 $\pm$ 1.5 %
77 h	19.2 $\pm$ 1.4 %
96 h	12.7 $\pm$ 1.6 %
100 h	11.2 $\pm$ 1.2 %

The effective half-life was consequently found to be 31.8 $\pm$ 0.7 hours and thus the biological half-life 11.7 $\pm$ 2.0 days.

As it was found that the bromide ions were not incorporated in fat or proteins of blood, it would seem that the amount of bromide ions inside the red cell membrane is probably distributed mainly in the intracellular water.

The steady state of Br^{82} in the blood was reached about 2—3 hours after the oral administration of the radioactive solution. Then the concentration of Br^{82} gradually decreased in an exponential course characterized by the biological half-life, which illustrated the continuous exchange of bromide ions in the body caused by different mechanisms. The bromide ions are eliminated from the blood mainly by excretion into the urine (SÖREMARK 1960). The amount of ammonium bromide administered to the subjects was considered to be sufficiently small not to disturb the physiological distribution.

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Some Observations on the Synthesis and Storage of Catecholamines in the Adrenaline Cells of the Suprarenal Medulla

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Abstract

Bertler, Å., N.-Å. Hillarp and E. Rosengren. *Some observations on the synthesis and storage of catecholamines in the adrenaline cells of the suprarenal medulla.* Acta physiol. scand. 1960. *50.* 124—131. — The intracellular distribution of dopamine, noradrenaline and adrenaline in rabbit suprarenal glands was examined by differential centrifugation of homogenates (0.3 M sucrose) of the medullary tissue. The amines were determined spectrophoto-fluorimetrically.

Glands from normal animals were found to contain no — or very small — amounts of dopamine and noradrenaline. When the medulla was stimulated to secretion by insulin-induced hypoglycemia, noradrenaline was rapidly formed. It accumulated in the "large granule" fraction, but largely disappeared together with the adrenaline store on prolonged stimulation. Large amounts of "bound" noradrenaline again accumulated during the recovery phase.

Very large amounts of dopamine and noradrenaline rapidly (within 30 min) appeared after an intravenous injection of L-dopa (100 mg/kg body wt). The new-formed amines became rapidly "particle-bound". In contrast to dopamine which largely disappeared after some hours the noradrenaline remained in large amounts for at least 24 hours.

A tentative scheme of the intracellular events which occur when the adrenaline cell synthesizes and stores catecholamines is proposed and discussed.

Abbreviations: Dopa = 3,4-dihydroxyphenylalanine, DA = dopamine, NA = noradrenaline, A = adrenaline.

The experiments described in this study were performed to obtain more information on some intracellular events which occur when the chromaffin cell synthesizes and stores catecholamines. A new approach to this problem now seems possible since it has been found that large amounts of DA and NA are rapidly formed in the adrenal medulla of rabbit after an intravenous injection of L-dopa (BERTLER, ROSENGREN and ROSENGREN 1960).

The suprarenal medulla of rabbit was used since this contains only adrenaline cells (*cf.* HILLARP and HÖKFELT 1953) and thus normally has no — or very small — amounts of NA. This is one of the prerequisites for the experiments. Another is that the synthesis of A takes place in the order: Tyrosine→Dopa→DA→NA→A. There is good evidence for this view (*cf.* BLASCHKO 1959).

Methods

The adrenal medulla of rabbit was stimulated to secretion by insulin-induced hypoglycemia (*cf.* HÖKFELT 1951). The animals were killed by an intravenous injection of air after different intervals during the secretion and recovery phases. The adrenals were immediately removed and chilled with ice. The medulla with some adhering cortical tissue was rapidly dissected and homogenized with a loose-fitting plastic pestle for about 20 sec in 7 ml of 0.3 M sucrose. To remove unbroken tissue and cells but at the same time to prevent losses of amine granules the homogenate was centrifuged at 800 × *g* for only 3 min. Two subfractions were obtained by centrifugation at 38,000 × *g* for 30 min: a) the "large granule" fraction, containing *i. a.* the amine granules and mitochondria, b) the fraction "cytoplasmic sap", containing the "soluble" cytoplasm and much microsomal material. All operations were done at 0° C and without any delay. The NA and A content in the two subfractions was determined spectrophoto-fluorimetrically (BERTLER, CARLSSON and ROSENGREN 1958, CARLSSON and WALDECK 1958). The low-speed sediment was also analyzed for NA and A and the degree of homogenization (usually 90 to 94 per cent) could thus be determined.

On the basis of the finding that intravenously injected L-dopa (100 mg/kg body wt) is rapidly converted to DA and NA in the normal adrenal medulla (BERTLER, ROSENGREN and ROSENGREN 1960) the content and distribution of DA, NA and A in the non-stimulated rabbit medulla were followed after such injections. — The animals in this series were killed by a blow on the head.

It is not possible to dissect the rabbit adrenal medulla without variable losses of medullary tissue. This is probably the explanation of the finding that the A content in the medulla of some normal or dopatreated animals was lower than that usually found. As has been observed both in these and other laboratories (*cf.* HÖKFELT 1951, KRONEBERG and SCHÜMANN 1957), however, the A content may vary markedly from rabbit to rabbit even if the entire glands are examined.

Results

The adrenal medulla of normal, untreated rabbits contained no, or very small, amounts of DA and NA (Table II).

As previously found by HÖKFELT (1951) NA is rapidly formed in the rabbit medulla when stimulated to secretion by insulin-induced hypoglycemia (Table I). This new-synthesized NA was not — as might be expected — recovered in

Table I. Content and intracellular distribution of adrenaline and noradrenaline in the rabbit's suprarenal medulla at varying periods of time after a subcutaneous injection of 5 IU insulin per kg body weight. Six hours after the insulin injection 20 ml of a 5% glucose solution were administered subcutaneously and the animals were given food ad lib. — Control animals are found in Table II.

"Free" amines represent the amines recovered in the high-speed supernatant and are expressed in per cent of the amines in the low-speed supernatant. Total amines are expressed in µg/kg body wt.

	Insulin 2 hr		Insulin 3½ hr		Insulin 6 hr		Insulin 6 hr Recovery 20 hr		Insulin 6 hr Recovery 46 hr	
	Total	"Free"	Total	"Free"	Total	"Free"	Total	"Free"	Total	"Free"
	µg	Per cent	µg	Per cent	µg	Per cent	µg	Per cent	µg	Per cent
Adrenaline	17	7	14	11	0.31	32	19	10	18	9
	31	9	5.6	20	0.79	22	14	11	11	11
	27	12	9.0	15			8.2	10	32	7
	17	8	8.0	12			27	7		
			3.5	19						
Noradrenaline	0.76	13	1.6	10	0.10	30	8.6	14	3.9	15
	1.5	15	0.80	30	0.07	100	7.4	7	6.2	8
	1.5	14	3.0	14			3.4	9	6.0	3
	6.4	2	0.85	12			3.8	9		
			0.55	25						

the cytoplasmic sap but was mainly "bound" to "particles" in the large granule fraction. It appeared before any outer signs of hypoglycemia had developed (2 hrs after insulin administration) and largely disappeared together with the A store on prolonged stimulation (6 hrs). Large amounts of bound NA again accumulated during the recovery phase in spite of new-synthesis and re-storage of A. Unfortunately DA was not determined in these experiments.

Already 20 to 30 min after an intravenous injection of L-dopa, large amounts of DA appeared in the medulla (Table II). The amounts produced must in fact be considerably higher than those observed since much of the DA was transformed to NA. On the basis of the figures observed for these amines at this point of time it may be calculated that the amounts of DA produced per hour may well be as high as 30 to 50 per cent of the amount of the stored A originally present in the glands. Table II further shows that NA also appeared with a rapidity and in amounts clearly indicating that the hydroxylation of DA occurred at a high rate. It may be calculated that the amounts of NA produced per hour could be as high as 30 to 40 per cent of the amount of the stored A.

The new-formed DA was mainly "particle-bound" already 20 to 30 min after the dopa administration. A considerably larger part of this amine than of A was, however, recovered in the fraction "cytoplasmic sap". Two hours after the dopa

Table II. Content and intracellular distribution of amines in the rabbit's suprarenal medulla at varying periods of the time after an intravenous injection of dopa (100 mg/kg body wt). "Free" amines represent the amines recovered in the high-speed supernatant and are expressed in per cent of the amines in the low-speed supernatant. Total amines are expressed in µg/kg body wt.

	Adrenaline		Noradrenaline		Dopamine	
	Total	"Free"	Total	"Free"	Total	"Free"
	µg	Per cent	µg	Per cent	µg	Per cent
Control	32	9	0.00		0.43	5
»	24	6	0.00		0.43	23
»	20	6	0.00		—	—
»	16	12	0.31	0	—	—
»	42	9	0.14	100	—	—
»	53	8	1.7	4	0.36	10
»	51	6	0.00		0.36	27
D, L-dopa 30 min ..	33	15	0.90	67	1.1	22
» 30 » ..	64	9	0.60	100	1.9	18
L-dopa 20 min	35	13	3.3	14	1.9	30
» 30 »	56	11	2.3	45	3.0	18
» 30 »	43	9	4.4	5	2.1	18
» 30 »	23	17	4.8	12	1.6	35
L-dopa 60 min	86	8	14	11	4.5	17
» 60 »	69	13	7.0	24	3.1	25
» 60 »	64	11	7.5	15	3.4	9
» 60 »	95	6	16	7	3.8	7
» 60 »	100	5	19	3	3.5	5
L-dopa 2 hr	55	9	8.0	15	0.38	8
» 2 »	57	7	1.9	32	0.90	8
» 2 »	78	6	6.0	12	1.1	7
L-dopa 24 hr	70	—	7.0	—	0.26	—
» 24 »	54	7	10	8	0.35	15
» 24 »	46	9	5.8	0	0.35	20
» 24 »	72	8	—	—	0.36	19

administration some DA was still present but after 24 hours it had largely disappeared.

The new-formed NA, on the other hand, showed partly a different distribution. In 3 of the short-term experiments it was mainly or to a large part recovered in the fraction "cytoplasmic sap" but in the other experiments most of it was found in the large granule fraction and thus showed approximately the same distribution as A. In contrast to DA, NA remained in large amounts 24 hours after the dopa administration.

Control experiments showed that only small amounts of DA appeared in the medulla after an intravenous injection of a very large dose (40 mg/kg) of DA.

There is thus no reason to believe that the DA found in the medulla after administration of dopa originated from DA formed elsewhere in the body.

Small amounts of DA, NA and A were found in the adrenal cortical tissue after dopa administration. The amines showed, however, the same mutual proportions as those in the medulla and thus must have been present in medullary cells in the cortex. This illustrates the difficulty of getting a complete separation of the two tissues in rabbit glands.

The distribution of DA within the cells of the kidney cortex was examined in some of the short-term experiments. Practically the whole amine content was recovered in the fraction "cytoplasmic sap".

Discussion

Controversial results have been obtained as regards the rate of amine synthesis and storage in the medullary cell (*cf.* EULER 1958). The slow re-accumulation of amines after a depletion has been thought to imply that the synthesis and/or storage processes are slow. Several experiments with direct determination of the secretion seem to show, however, that the synthesis may proceed at a high rate, at least during periods of high secretion (HÖKFELT and MCLEAN 1950, HOLLAND and SCHÜMANN 1956, BYGDEMAN and EULER 1958), but others failed to confirm this (BUTTERWORTH and MANN 1956, EADE and WOOD 1958).

The present experiments show that the chromaffin cell may display activities in these respects that are much higher than those generally assumed. NA is formed at a high rate in the medulla during secretion. Since during periods of low secretory activity the medullary cell does not contain NA, this may mean that stimulation increases the rate of synthesis. The experiments also show however, that the cell during "resting" conditions may rapidly synthesize both DA and NA when dopa reaches the cell from outside. This clearly indicates that both the decarboxylation and hydroxylation enzymes in the "resting" cell have sufficient activites to make possible a high synthesis rate but that, on the other hand, they do not work at their maximal capacity owing to lack of substrates. The increased rate of NA synthesis during secretion thus seems to be made possible by the fact that stimulation accelerates the production of dopa in the cells (or possibly the uptake of dopa or its precursors from outside). The rate-limiting step must probably be located somewhere in the initial part of the synthesis sequence. Here, probably, is the mechanism which regulates the synthesizing activities of the chromaffin cell.

It has been proposed that the introduction of the side-chain hydroxyl group is the rate-limiting step in NA formation (BLASCHKO 1959). The results obtained in this study indicate that this may be the case when the cell is provided with large amounts of dopa from outside, but they also show that hydroxylation may proceed much more rapidly than might be expected from the *in vitro* experiments made by DEMIS, BLASCHKO and WELCH (1956). During "resting" conditions the

rate of amine synthesis is probably low but — as pointed out above — this seems to be due to a slow production (or uptake) of dopa.

The injection of dopa may cause central sympathetic stimulation and thus a stimulation of the adrenal medulla. It seems unlikely, however, that the DA and NA formation observed after dopa administration should be caused in this indirect way. Experiments in progress with denervated glands clearly support this view.

On the basis of the findings in the present work and of results obtained in other laboratories (see below) concerning the sequence and site of amine synthesis in the medullary cell, the following scheme may tentatively be proposed and discussed.

a) That dopa is probably formed from tyrosine has been shown by UDENFRIEND *et al.* (1953), UDENFRIEND and WYNGAARDEN (1956) and GOODALL and KIRSHNER (1957). It is not known, however, where the synthesis site is located in the cell. This step or some step previous to this seems to be the rate-limiting step which is accelerated during secretion.

b) The decarboxylation of dopa to DA probably occurs in the "cytoplasmic sap" — in its broadest sense — since BLASCHKO, HAGEN and WELCH (1955) have found the dopa-decarboxylase in this part of the cell. This agrees well with our finding that DA formed from dopa in the kidney cortex is recovered in the "cytoplasmic sap". It is unfortunately difficult to interpret the observation (SCHÜMANN 1958) that DA in bovine splenic nerves is recovered in the "soluble" cytoplasm, since the DA may have originated from the special dopamine cells occurring in these nerves (BERTLER *et al.* 1959). Further, the splenic nerves are very difficult to homogenize.

The new-formed DA is rapidly "bound" in some way in or to particles in the large granule fraction. The hydroxylation system is present in this fraction (KIRSHNER 1957) and this may afford an explanation of the finding that the DA becomes "particle-bound". On the other hand, in rabbits treated with reserpine, which presumably destroys the amine storage mechanism (*cf.* CARLSSON *et al.* 1957), the DA and NA formed from injected dopa have mainly been recovered in the "cytoplasmic sap". This favours the view that DA in fact accumulates in the storage granules. The storage mechanism may well be quite unspecific, a view which finds further support in the observation that 5-hydroxytryptamine formed from 5-hydroxytryptophane in the adrenal medulla also accumulates in the large granule fraction (BERTLER, ROSENGREN and ROSENGREN 1960).

c) The DA is rapidly hydroxylated to NA. Since the DA hydroxylating system is located in the particulate fraction (KIRSHNER 1957) it might be — as suggested by BLASCHKO (1959) — that the amine granules are actually the site of NA formation. The data hitherto obtained do not fit in too well with this view, however. It is not supported by the fact that new-formed NA during the first 30 min after dopa administration in some of the experiments to a large extent

was recovered in the "cytoplasmic sap". If the DA were hydroxylated in the storage granules it seems unlikely that the new-formed NA should escape the apparently highly efficient storage mechanism (see below). Furthermore, since the methylation of NA to A probably occurs somewhere outside the storage granules (see below) it might be expected — if the view mentioned above is correct — that the amine granules normally should contain some NA which is generally not the case.

d) There seem to be two alternative pathways for the new-formed NA. At least at high synthesis rates, large amounts of the amine rapidly accumulate in the large granule fraction. The finding that large amounts of "bound" NA still remain 24 hours after a single dopa injection and the reserpine experiments mentioned above clearly support the view that the NA in fact is incorporated in the storage granules. The storage mechanism apparently cannot choose between A and NA and its efficiency seems to be high, in fact so high that it competes with the methylating system. This seems to be the most reasonable explanation of the puzzling fact that the cell, which normally only contains A, may accumulate large stores of NA after dopa injection or in the recovery phase after a depletion. It also affords a good explanation of the interesting finding that new formed NA, after an amine depletion in rat and cat medulla, may remain for more than a week (BUTTERWORTH and MANN 1957, CALLINGHAM and MANN 1958). This is in good accordance with the slow turnover of stored amines as found by UDENFRIEND and WYNGAARDEN (1956). What happens to this stored NA is unknown but it seems reasonable that it is slowly released, methylated and then secreted or re-stored.

The other path is methylation to A which — as KIRSHNER and GOODALL (1957) have shown — probably occurs somewhere in the cytoplasm outside the storage granules. The present experiments do not show whether new-formed NA may be directly converted to A but this seems reasonable. It is also supported by experiments of MASUOKA *et al.* (1956) which showed that injected C^{14}-noradrenaline is rapidly converted to adrenaline in the adrenal medulla *in vivo*. The final step, then, is the storage process.

The scheme discussed above is, however, not more than a first attempt to get an idea of the intracellular events in the chromaffin cell. It must also be observed that these events probably occur in a highly organized cytoplasm. The denotion "cytoplasmic sap", for instance, must therefore not be taken too literally.

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Acta physiol. scand. 1960. 50. 132—139

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The Retention of Parenterally Injected Calcium in Rachitic Dogs

By

FREDRIK C. GRAN

Received 25 May 1960

Abstract

GRAN, F. C. *The retention of parenterally injected calcium in rachitic dogs.* Acta physiol. scand. 1960. *50.* 132—139. — The calcium and phosphorus balances were studied in two vitamin D free, rachitic dogs, 8 months old, given diets rich in Ca and P for 121 days. In the course of the last 113 days 36.4 g Ca (Ca glucono-galacto-gluconate) was injected parenterally. Practically all the injected Ca was retained. The faecal Ca exceeded food Ca by about 80 mg daily; the daily urinary Ca was 20—30 mg. The dogs gained about 20 g in body Ca in the period of injections. The plasma Ca level was about 7.8 mg/100 ml and was unaffected by the Ca injections. Inorganic P in serum was about 11.5 mg/100 ml before the injections of Ca were started; following the daily Ca load, serum P was reduced to approximately 4 mg/100 ml. At the end of the experiment one dog received vitamin D. The result was an increase in plasma Ca to 10.7 mg/100 ml in the course of 48 hours and a simultaneous reduction of the urinary Ca. Thus, vitamin D possibly affects the tubular reabsorption of Ca.

NICOLAYSEN and JANSEN (1939) found normal values for the bone ash in vitamin D free rats in experiments designed to give equal supply of Ca and P to the blood in rats with and without vitamin D. However, it has frequently been maintained that rickets in rats do not correspond to the rickets in children, in puppies, etc. Many have doubted that the results achieved in rats can be applied to other species. It was therefore decided to perform experiments in vitamin D free puppies. The aim was to observe if an uncalcified matrix of a rachitic puppy would accrete bone salts as avidly as vitamin D free rats do.

Methods

The faeces collected in the balance experiments were dried in vacuum on a steam bath, weighed, and powdered. The urines were preserved with nitric acid in the collecting bottles. Aliquots of faeces and urine were prepared for determinations of Ca and P by digestion with nitric and perchloric acids.

The Ca determinations were carried out in the conventional manner by titration of Ca oxalate with potassium permanganate. The perchlorates present did not interfere with the Ca determinations. Ca in blood serum was determined by the method of GRAN (1960 a). The P determinations were carried out by the method of FISKE and SUBBAROW (1925); blood serum was first deproteinized with trichloracetic acid. The dietary intake in the metabolic periods was determined with 0.5 per cent chromic oxide marker in the diet (GRAN 1960 b). All analytical determinations were carried out at least in duplicate.

An approximately isotonic solution of calcium glucono-galacto-gluconate (Sandoz A. G., 70.5 mg Ca per g) was sterilized for 30 minutes at 120° in small flasks. Fifty ml of the sterile solution contained 200 mg Ca. The flasks were stored in the cold and were heated to body temperature before the solution was injected. Daily injections were given subcutaneously in the back of the dogs under aseptic conditions.

Procedures

Two mongrel female dogs from a litter of seven were used in the experiments to be reported, however, these dogs were not selected until all the puppies had developed rickets. The mother received a liberal supply of cod-liver oil in gestation and lactation. The puppies were weaned at the age of three weeks and were separated from their mother one week later. Following weaning, the puppies were each given orally 70 I. U. vitamin D_2 weekly until the age of three months, when the administration of vitamin D was discontinued. The puppies were not exposed to sunlight.

Following weaning the puppies received a vitamin D free diet of the following composition: 5 per cent evaporated skim milk powder, 2 per cent crystalline egg albumen, 10 per cent acid precipitated casein, 10 per cent arachis oil, 10 per cent sucrose, 57 per cent ground whole wheat, 2 per cent dried brewer's yeast, 1 per cent salt mixture, 2 per cent potassium phosphate (KH_2PO_4), 1 per cent calcium carbonate, and 0.05 per cent choline chloride. In addition, each kg diet contained 10 μg vitamin B_{12}, 2 mg menadione and 10,000 I. U. vitamin A acetate. The diet contained 0.5 per cent Ca and 0.6 per cent P. Adjustments were made in the dietary content of ground whole wheat when other additions to the diet were required. The dietary components were mixed well and stored in the dry state. A porridge was cooked daily for a few minutes. Food and water was allowed without restriction.

The salt mixture contained 60 per cent potassium chloride, 33.1 per cent sodium chloride, 2.2 per cent magnesium sulphate, 4 per cent ferric chloride, 0.5 per cent potassium iodide, 0.1 per cent copper sulphate and 0.1 per cent manganese acetate.

The development of rickets was followed by radiological examination of the distal ends of ulna and radius and by determinations of Ca and P in blood serum. At the age of five months, five of the puppies were distinctly rachitic as indicated by X-ray and low Ca and high inorganic P in serum. The dogs were kept for another three months on the diet before the two experimental animals were selected. The result was a very well developed rickets as judged also by various clinical signs.

The balance experiments were next started and the dogs were kept in their individual cages throughout the experiment.

Table I. The retention of Ca and P in dog number 1 given additional Ca parenterally by

Period No.	Duration days	Body wt. kg	Serum mg/100 ml		Food eaten g/day	Ca injected mg/day	Calcium in mg/day	
			Ca	P			oral intake	absorbed
[1]1	2	12.0	8.0	13.5	486	—	2,419	— 97
2	3				268	—	1,335	—182
3	3	13.0	8.1	11.3	261	—	1,297	10
[2]4	4	14.1	7.8	10.7	88	200	488	— 75
[3]5	4				167	200	890	—106
6	4		7.6	5.3	166	200	882	—131
7	4				159	200	844	—100
8	7		6.9	4.4	157	200	834	— 80
9	7		6.4	4.3	142	200	755	— 54
10	7	10.3	6.7	4.9	110	200	587	— 80
11	7		6.8	3.7	147	200	784	— 41
[4]12	12	11.1	7.1	4.1	134	400	689	— 47
13	12	11.9	8.4	4.7	179	400	920	— 85
14	12	12.1	8.2	6.8	188	400	965	—143
15	12		7.2	5.3	196	400	913	—132
16	12	12.4	7.2	6.0	170	400	789	—120
[5]17	9	12.0	10.7	4.9	181	400	933	472

[1] 0.498 per cent Ca and 0.630 per cent P in the diet, the balances were taken 6 weeks
[2] 0.556 per cent Ca and 0.625 per cent P in the diet, 200 mg Ca was injected daily as
[3] 0.532 per cent Ca and 1.160 per cent P in the diet, 200 mg Ca was injected daily in
[4] 0.514 per cent Ca and 1.175 per cent P in the diet, 400 mg Ca was injected daily in
[5] 0.465 per cent Ca and 1.201 per cent P in the diet, 400 mg Ca was injected daily in

A very rachitic female mongrel dog was used in the experiments. The dog was $6^1/_2$ months started. Blood for determinations of serum Ca and P was taken on the first day of each obtained 48 hours after the administration of vitamin D.

The two puppies used weighed 10.5 and 10.8 kg at the age of five months, the body weights of dogs no. 1 and no. 2 three months later were 14.1 and 11.7 kg, respectively.

Experimental

The Ca balances of the dogs were measured about six weeks in advance of the experiments which followed. Two to four days passed between each metabolic period in this phase of the experiments. The diet contained 0.498 per cent Ca and 0.630 per cent P. Ca was not given parenterally in these periods.

Two hundred mg Ca was injected once daily for the following 44 days. Next the injected amount of Ca was doubled (one injection daily) in the following 69 days. The Ca content of the diets was kept constant throughout the experiments while the P content was doubled as indicated in Table I and II.

daily subcutaneous injections

Calcium in mg/day		Total Ca retained in experiment mg	Phosphorus in mg/day				Total P retained in experiment mg
urine	retained		oral intake	absorbed	urine	retained	
32	—129		3,060	1,228	771	457	
13	—195		1,689	662	833	—171	
10	0		1,641	700	641	59	
14	111	444	548	154	173	— 19	— 86
23	71	728	1,940	1,103	700	403	1,526
17	52	926	1,924	1,109	674	435	3,266
16	84	1,524	1,841	1,152	587	565	5,526
63	57	1,923	1,819	1,080	1,209	—129	4,623
17	129	2,826	1,647	1,054	740	314	6,821
17	103	3,547	1,281	788	800	— 12	6,737
21	138	4,513	1,709	1,043	841	202	8,151
17	336	8,545	1,574	991	921	70	8,991
18	297	12,108	2,103	1,213	919	294	12,519
61	196	14,459	2,205	1,221	1,433	—212	9,975
46	222	17,124	2,358	1,360	1,243	117	11,379
97	183	19,509	2,476	1,600	1,398	202	14,005
28	844	27,104	2,636	1,710	1,390	320	16,885

prior to the following experimental periods which were conducted successively.
Ca glucono-galactogluconate.
one dose.
one dose.
one dose. 5,000 I. U. vitamin D_2 was given on the first day of the period.

old when taken into the experiments, and was about 8 months old when period 4 was
period before Ca was injected, with the exception that the values given for period 17 were

In the final period dog no. 1 (Table I) received one dose of 5,000 I. U. vitamin D_2 orally on the first day, whereas dog no. 2 (Table II) was retained in the experiment as a vitamin D free control.

Results

A. The Ca and P balances. The results are given in Table I and II. Periods 1—3 represent observations made six weeks prior to the actual experiments. Some variations occurred in the daily food intake and are reflected in the variability in the Ca and P intake in the various periods. A reduction in the daily food intake was observed after the start of the Ca injections, and a loss of body weight was also observed.

Table II. The retention of Ca and P in dog number 2 given additional Ca parenterally by

Period No.	Duration days	Body wt. kg	Serum mg/100 ml		Food eaten g/day	Ca injected mg/day	Calcium in mg/day	
			Ca	P			oral intake	absorbed
[1]1	2	11.1	8.4	12.1	356	0	1,772	— 88
2	3				231	0	1,149	—110
3	3	11.1	7.8	10.8	245	0	1,222	— 42
[2]4	4	11.7	7.1	10.4	98	200	544	— 65
[3]5	4				122	200	651	— 97
6	4		7.9	3.9	149	200	791	—121
7	4				118	200	625	— 96
8	7		6.9	4.3	133	200	709	— 59
9	7		6.4	4.0	126	200	670	— 79
10	7	9.7	6.5	3.9	84	200	445	— 62
11	7		5.9	4.8	162	200	861	— 26
[4]12	12	9.8	6.1	4.6	175	400	898	— 79
13	12	10.0	7.7	5.0	226	400	1,160	—108
14	12	10.0	8.5	7.4	186	400	957	—102
15	12		7.1	4.9	188	400	874	—145
16	12	10.3	7.7	4.1	174	400	809	—167
17	9	10.6	8.2	4.6	196	400	1,013	— 60

[1] 0.498 per cent Ca and 0.630 per cent P in the diet, the balances were taken 6 weeks
[2] 0.556 per cent Ca and 0.625 per cent P in the diet, 200 mg Ca was injected daily as
[3] 0.532 per cent Ca and 1.160 per cent P in the diet, 200 mg Ca was injected daily in
[4] 0.514 per cent Ca and 1.175 per cent P in the diet, 400 mg Ca was injected daily in

A very rachitic female mongrel dog was used in the experiments. The dog was $6^1/_2$ months started. Blood for the determinations of Ca and P was taken on the first day of each

The faecal excretion of Ca varied with the Ca intake; however, the net absorption was continuously negative, as expected in advanced vitamin D deficiency in puppies (MELLANBY 1949). The injections of Ca did not result in any measurable increases in the faecal Ca excretion. This observation is fully in line with the current concepts.

It appears that the urinary Ca was low in the early periods of the injections and was not at all influenced by the Ca load. However, in the later periods the urinary Ca was increased, as was to be expected. Although the urinary Ca varied considerably from period to period, the clear-cut result is that by far the greatest proportion of the injected Ca was retained. In the last periods of the experiments the retention seems to have been somewhat reduced.

As regards the P balance it is clear that an increase in the P retention

daily subcutaneous injections

Calcium in mg/day		Total Ca retained in experiment mg	Phosphorus in mg/day				Total P retained in experiment mg
urine	retained		oral intake	absorbed	urine	retained	
57	—145		2,242	930	1,047	—117	
59	—169		1,453	631	681	— 50	
10	— 52		1,545	656	674	— 17	
15	120	476	612	204	185	19	86
18	85	816	1,420	870	626	244	1,062
19	60	1,056	1,725	1,010	599	411	1,706
24	80	1,623	1,364	830	511	319	3,982
65	76	2,155	1,546	929	1,058	—129	3,078
20	101	2,862	1,460	881	717	164	4,226
8	130	3,772	970	597	622	— 25	4,051
4	160	4,892	1,878	1,085	748	337	6,410
16	305	8,554	2,053	1,160	869	291	9,902
17	275	11,855	2,653	1,620	1,155	465	15,482
38	260	14,979	2,188	1,221	1,710	—489	9,614
60	195	17,322	2,257	1,324	1,290	34	10,022
86	147	19,237	2,540	1,380	1,442	— 62	9,216
43	297	21,906	2,860	1,511	1,336	175	10,791

prior to the following experimental periods which were conducted successively.
Ca glucono-galactogluconate.
one dose.
one dose.

old when taken into the experiments, and was about 8 months old when period 4 was
period before Ca was injected.

followed the injections of Ca, however, the variability was considerable and in a few periods negative P balances occurred simultaneously with positive Ca balances.

B. The effect of vitamin D. In the final period vitamin D was given to dog no. 1 (Table I). The result is a striking increase in the absorption and retention of Ca and P, and is fully in line with the expectations according to numerous earlier observations by a variety of investigators (NICOLAYSEN and EEG-LARSEN 1953).

C. Serum Ca and P. The serum Ca was low, as expected and it was not increased following the daily Ca load. The blood was sampled before the injection; however, in some instances blood was sampled at hourly intervals

after the injection. The disappearance of the subcutaneous pad was fairly rapid, about 2 hours, and no increase in serum Ca parallel to the disappearance could be observed. Such a result seems to be in accord with the non-influence on serum Ca of the slow intravenous Ca infusion of WOLF and BALL (1949).

Following the vitamin D administration to dog no. 1 the serum Ca increased to normal, as expected, in the course of a couple of days. Actually the normal serum Ca was observed 48 hours after the dose of vitamin D.

The inorganic P in the serum was high before the injections of Ca, which reduced the values by about 2/3, the value remained at a level following the vitamin D administration. In short, vitamin D normalized the Ca $\times$ P product.

D. Physical condition. Finally, a few comments are appropriate on the physical condition of the dogs. They suffered from a very severe rickets when they were taken into the experiments. The radiological examination demonstrated a wide uncalcified epiphyseal cartilage, and the bones were also severely deformed. In consequence, the dogs were physically very inactive. Their condition improved remarkably when Ca had been injected for several weeks; towards the end of the experiments the dogs were much more active. The epiphyseal cartilage was narrow and fully calcified at the end of the experiments, in fact it was reduced to a very narrow slit, just visible.

Discussion

The total Ca retention following the Ca ingestion was nearly 20 g in both dogs. The result in dog number 1 following vitamin D administration, however, indicates that additional retention could be achieved. On the other hand such dogs would, in a normal state, be expected to contain about 100 g Ca. The retention of about 20 g thus corresponds to about 20 per cent of this value.

The P retention was such that the value for Ca/P retained was 1.61 and 2.03, respectively, in the two dogs. Most analyses of bones indicate a value of 2.2 for Ca/P in ashed bone. Since the dogs maintained a fairly constant body weight, no explanation can be given.

Possible kidney effect of vitamin D. Following the administration of vitamin D, plasma Ca increased from 7.2 up to 10.7 mg/100 ml in the course of 48 hours. Simultaneously the Ca excretion in the urine decreased from 97 to 28 mg per day. Unfortunately clearance studies were not performed, but the results are indicative of an effect of vitamin D on the tubular reabsorption of Ca. The results are in full conformity with those observed in rats (NICOLAYSEN and EEG-LARSEN 1956). In both instances a simultaneous depression of the volume of the glomerular filtrate by about 40 per cent would be needed to explain the observed values. The phosphate absorption was little changed following the vitamin D administration and it is therefore not likely that the depression of Ca excretion in the urine is the result of an increased phosphate load (MALM 1953).

Attention should be drawn to the effect of vitamin D on citrate metabolism in the kidney (DE LUCA, GRAN, and STEENBOCK 1957, DE LUCA *et al.* 1957). Thus evidence is accumulating in favour of the view that vitamin D has a general metabolic effect. However, it is still the view of this Department that the rachitic lesion, viz. the faulty bone formation, is chiefly due to the reduced speed of absorption of Ca. In fact the work here presented in dogs substantially supports such a view.

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Acta physiol. scand. 1960. 50. 140—152

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Some Characteristics of the Hypothalamic "Drinking Centre" in the Goat as Shown by the Use of Permanent Electrodes

By

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Abstract

Andersson, B., S. Larsson and N. Persson. *Some characteristics of the hypothalamic "drinking centre" in the goat as shown by the use of permanent electrodes.* Acta physiol. scand. 1960. *50.* 140—152. — The response to electrical stimulation of the "drinking centre" was studied in one goat for a period of six months. Emotional factors and hydration were found to increase the stimulus threshold for drinking. Drinking continued for different lengths of time after cessation of stimulation. The after-effect which may be due to an after-discharge in the stimulated area of the hypothalamus was inhibited by the previous injection of amphetamine or the drinking of cold water. On repeated stimulations the animal learned to overcome a simple obstacle in order to gain access to the drinking water. If the goat was not allowed to drink, the stimulations elicited a typical "displacement reaction". Prolonged strong stimulations of the "drinking centre" were not observed to alter the electrolyte metabolism significantly.

Previous experiments in which osmotic and electrical stimuli were applied to the hypothalamus for studies of central regulation of water intake were all of relatively short duration (Andersson 1952, 1953, Andersson and McCann 1955 a and b, Greer 1955). The stimulation technique used did not permit studies for more than a few days in one and the same animal. Consequently a more thorough analysis of the response to stimulation of the "drinking centre" under varying physiological conditions has heretofore not been made.

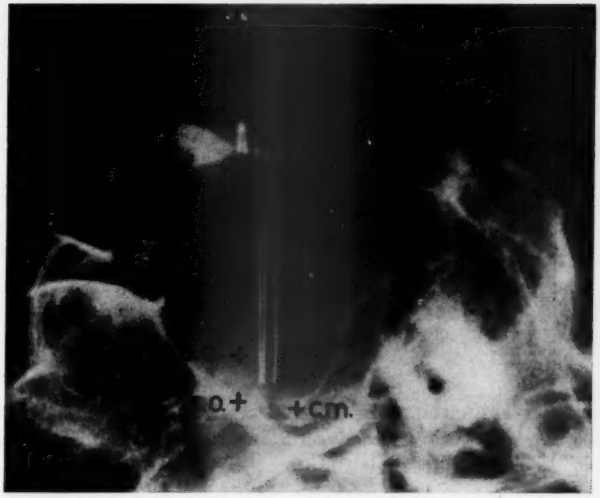

Fig. 1. An X-ray picture of the skull of the goat showing the three electrodes implanted into the hypothalamic "drinking area".
c. a. = approximate position of the anterior commissure.
c. m. = » » » mammillary body.
c. o. = » » » optic chiasma.

The use of permanent platinum-iridium electrodes implanted into the hypothalamus made it possible to study the effect of electrical stimulations of the "drinking centre" in the same animal for a period of six months. In the following the results of such a study are reported.

Methods

The experiments were performed with an adult, female goat (body weight: 35 kg), which had three electrodes implanted into the hypothalamic "drinking area" on Oct. 13th, 1959. In an acute stimulation experiment functional localization was used as an aid for the correct placement of the electrodes. The method of implantation and the technique of electrical stimulation has been described previously (ANDERSSON, PERSSON and STRÖM 1960). In all experiments except those made as special tests of the effect of different stimulus parameters, the pulse width was 3 msec and the pulse frequency 50 c. p. s. The stimulus strength varied between 0.05 and 0.6 mA. Providing an adequate stimulus was applied, a drinking response was obtained due to unipolar stimulation via each one of the three hypothalamic electrodes (named A, B and C in rostro-caudal order) or due to bipolar stimulation between any two of the electrodes. The resistance of the electrodes *in situ* were: A = 5 kΩ, B = 4.2 kΩ and C = 3.5 kΩ. Fig. 1 is an X-ray picture of the head of the goat showing the positions of the three hypothalamic electrodes. During placement of the electrodes, B was implanted slightly off centre so that its uninsulated tip came closer to the tip of electrode A than to that of C.

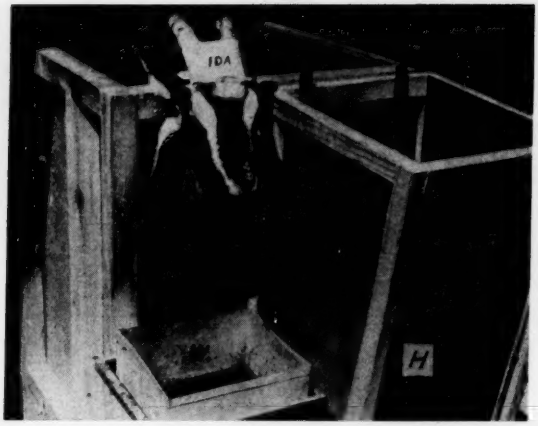

Fig. 2. The goat placed in its costumary environment (a metabolism cage) where most o the experiments were performed.
H = Foddering-rack.
W = Water container.
An aluminium shielding round the horns is protecting the electrodes.

During the six months of study the goat was kept collared in a metabolism cage (Fig. 2). On rare occasions stimulation experiments were made in another environment. In the metabolism cage the animal generally had free access to water and was fed a constant diet (1.5 kg hay and 250 g groats with 4 g NaCl added) once a day at 10.00. The goat gave birth to a kid on Jan. 18th 1960 which was weaned 10 days later. Since then the goat was milked once a day at feeding time. During periods of balance studies the urine was collected every 24 hours and analysed for chloride according to BRUN's (1949) method and for sodium and potassium using the EEL flame photometer. The same methods were used for determining plasma chloride, sodium and potassium. Blood sugar determinations were made according to the method of SOMOGYI (1945). During periods of balance studies the daily intake of water and the milk yield were also recorded.

Stimulation experiments to test the drinking response were generally made at certain times of the day, starting either at 9.30 A. M. (before feeding) or at 13.00 P. M.

Results

A. General features of the drinking response to stimulation

During the acute stimulation experiment, preceding the permanent fixation of the electrodes, the animal responded to a weak unipolar stimulation via electrodes B and C (0.05 mA, 3 msec, 50 c. p. s.) with the drinking of tepid water. Stronger stimuli applied via these electrodes or via electrode A caused restlessness which seemed to detract from drinking. No further test was made until a month later. The goat was then stimulated in its normal environment and much stronger stimuli than in the acute experiment could then be applied

Table I. Stimulus thresholds for positive drinking response during the period Nov. 23rd 1959 to May 11th 1960

A → 0 mA	B → 0 mA	C → 0 mA	A → B mA	B → C mA
0.2 (7)	0.3 (11)	0.15 (2)	0.15 (13)	0.15 (2)
0.3 (2)	0.4 (3)	0.2 (8)	0.2 (9)	0.2 (4)

The drinking response was considered positive if the animal drank water at 12° C within 10 sec after beginning of stimulation on repeated stimulations. The number of tests in brackets. All tests were performed in the goat's normal environment and under standardized conditions.
A, B and C = Hypothalamic electrodes.
0 = Indifferent electrode.
Pulse width = 3 msec, Pulse frequency = 50 c.p.s.

via all three electrodes without causing obvious restlessness. Unipolar stimulation of moderate strength via each one of the electrodes or bipolar stimulation between any two of the electrodes induced the drinking of cold water. The stimulus threshold for positive drinking response was dependent upon whether unipolar or bipolar stimulation was used and also upon via which electrode the stimulus was applied. The goat was placed in the metabolism cage where with occasional exceptions it has been kept until today. More than 500 stimulations of varying duration and strength were applied during the six month period of study. In spite of this fact the stimulus threshold for positive drinking response remained remarkably stable, if the internal and external conditions of the animal were kept relatively constant. The constancy of the response to stimulation was illustrated in a previous publication (ANDERSSON *et al.* 1960) and is shown in Table I.

In tests made for determination of the stimulus threshold for a drinking response, water with a temperature of about 12° C was offered and the response was considered positive providing the animal on repeated stimulations started to drink within 10 sec from the beginning of stimulation. In order to avoid over-drinking the stimulation was discontinued soon after the goat had started to drink. "Subthreshold" stimuli were often seen to induce drinking after longer latency periods than 10 sec, but at times the primary stimulus induced drinking within less than 10 sec. The latter effect of a weak stimulus was, however, generally not seen again on repeated stimulations. "Threshold" or slightly stronger stimuli as a rule caused drinking within 1 to 4 sec and the effect could be repeated at will.

After stimulation drinking continued for different lengths of time. Providing the temperature of the water was constant, the duration of the after-effect was to a certain degree dependent upon the duration and the strength of stimulation. Drinking generally stopped immediately after cessation of stimulations of moderate strength and of short duration. Relatively strong stimulations

of long duration (20 to 40 sec) were on the other hand seen to cause drinking persisting as long as 25 sec after stimulation. When the stimulation lasted for 15 sec or longer, the animal was prevented from over-drinking by covering the container with a lid which made the water inaccessible. Free access to water was under such circumstances allowed 5 to 10 sec before end of stimulation. In some experiments, however, the lid was not removed until just after the stimulation was discontinued. Providing the goat had been stimulated with a relatively strong stimulus of long duration (30 to 40 sec), it might then start to drink and continue drinking for up to 15 sec, although no longer stimulated (Fig. 3 D). The after-effect due to a certain stimulus gradually diminished on repeated stimulations. Other factors influencing the duration of the after-effect are described below.

"Subthreshold" stimuli were generally seen to induce licking and biting the walls of the cage and other objects within the reach of the goat. "Threshold" or slightly stronger stimuli, applied when a lid was loosely placed over the water container, caused the goat to bite and push the lid and elicited the "displacement reaction" described below. After relatively few repeated stimulations the animal learned to push the lid aside and obtain water, and then the stimulations no longer elicited any "displacement reaction".

Strong stimuli applied unipolarly via electrode A (0.5 mA) or bipolarly between electrodes A and B (0.4 mA) caused rage reactions which often led to attack directed towards persons coming close to the goat. The rage reaction generally detracted from drinking, but if the goat once started to drink due to a rage provoking stimulus, it drank ravenously.

B. External and internal factors changing the drinking response.

a) *Emotional factors:* If the goat was taken from its normal environment (the metabolism cage) and was placed in a pen in the laboratory, the stimulus threshold for positive drinking response was increased considerably. In addition, the latency period before onset of drinking was longer than that seen when the same stimulus was applied in the normal environment of the goat. However, repeated stimulations in the unfamiliar environment gradually lowered the stimulus threshold for a drinking response and shortened the latency period before onset of drinking. The same type of facilitation of the drinking response to stimulation of the "drinking area" was observed in previous acute stimulation experiments in other goats.

Before the newborn kid was weaned, it was sometimes present in the metabolism cage during stimulation experiments. A stimulus of "threshold" strength then often elicited the "displacement reaction" described below, but no drinking. The stimulus threshold for a drinking response was about twice as high as that observed before the kid was let into the cage. When the kid was sucking, still stronger stimuli had to be applied on repeated occasions before the mother goat was induced to drink.

b) *Hydration:* The stimulus threshold for positive drinking response was determined on three occasions two hours after the goat had received 5 liters of warm water by stomach tube, and was compared to that observed before and the day after hydration. Hydration of this magnitude caused a drop of plasma sodium of approximately 4 meq/l and of plasma chloride of about 6 meq/l. Although polydipsia still readily could be induced by electrical stimulation of the "drinking centre", the stimulus threshold for positive drinking response was approximately twice as high as that observed before hydration or on the following day. Even after prolonged stimulations the after-effect was very short during hydration.

c) *The temperature of the accessible water:* Goats normally seem to prefer warm water to cold. Tests in which several goats had free access to water at 12° C showed that they spontaneously drank half a liter or more if water at 30° C was offered to them. Studies of drinking after previous dehydration have also shown that goats offered water at 1° C drink only about two thirds of the amount they drink when offered tepid water (ANDERSSON, JEWELL and LARSSON 1958). It therefore seemed to be of interest to study whether or not different temperatures of the water influenced the response to electrical stimulation of the "drinking centre". To obtain a reliable answer to this question a great number of tests were made on different days. When the goat became aware that the accessible water was warm, the drinking response seemed to be facilitated, as indicated by a somewhat lowered stimulus threshold for a positive drinking response. Much more conspicious, however, was the effect of changes of the water temperature on drinking persisting after stimulation. If the goat had access to water at 3° C, it stopped drinking immediately on cessation of stimulation, even when the stimulus applied was strong and of long duration. When the accessible water had a temperature of 32° C, drinking continued for considerably longer periods of time after stimulation than due to the identical stimulus applied when water at room temperature was offered.

d) *Amphetamine:* This drug was found to inhibit drinking normally occurring after the intravenous injection of hypertonic saline in the dog (ANDERSSON and LARSSON 1956). Its inhibitory effect on drinking in the goat was found to be less obvious (ANDERSSON *et al.* 1958). Determinations of stimulus threshold for positive drinking response and studies of the duration of the after-effect was made on four occasions half an hour after the intramuscular administration of amphetamine (Phenopromin, ACO, 1.5 to 1.8 mg/kg b. wt.). The results were compared to those of similar tests made before the administration of the drug. The stimulus threshold for drinking was not significantly altered by amphetamine given in these doses. However, under the influence of amphetamine the animal stopped drinking immediately on cessation of stimulation, even after a strong stimulus of long duration and even if water at 32° C was offered.

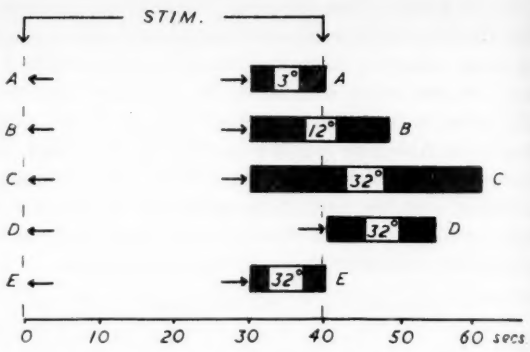

Fig. 3. A diagram to show how different factors influenced the duration of drinking persisting after electrical stimulation of the hypothalamic "drinking centre".
The black rectangles indicate the periods of drinking. The goat had no access to water during the period limited by the horizontal arrows.
A = Offered water at 3° C
B = » » » 12° C
C = » » » 32° C
D = » » » 32° C
but not allowed access to water until the end of stimulation.
E = Offered water at 32° C under the influence of amphetamine.
Stim. = The period of electrical stimulation. Unipolar stimulation via electrodes A and B. Strength = 0.4 mA; Pulse width = 3 msec; Pulse duration = 50 c. p. s.

Fig. 3 illustrates changes in the duration of the after-effect due to different temperatures of the accessible water and due to the administration of amphetamine.

e) Other drugs: Determinations of stimulus threshold for positive drinking response was made on two occasions 15 min after the intravenous injection of atropine (atropine sulfate, 0.3 mg/kg b. wt.). Similar tests were made 5 and 10 min after the intravenous injection of ephedrine (1.2 mg/kg b. wt.). These drugs were not observed to change the stimulus threshold for drinking. The effect of certain "psycholeptic" drugs is described in the following.

C. A "displacement reaction" elicited by stimulation of the "drinking centre"

During stimulations of long duration the animal was generally not allowed access to water until towards the end of the period of stimulation. To prevent the goat from drinking, a lid which it could not remove covered the water container. Due to a "threshold" or stronger stimulus the goat moved towards the water container and tried in vain to push the lid aside. When finding itself unable to gain access to the water, it showed signs of distress and started to scratch the abdominal wall vigourously with the left hind leg. If the stimulus was not discontinued, the scratching went on for 3 to 5 sec. A second period of scratching often appeared after another 5 to 10 sec if the stimulation was continued. If the goat during a second period of stimulation again had free access to water, it as a rule started to drink with no signs of distress and with no scratching. However, three or more repeated stimulations with the water container closed had the effect that during the next stimulation performed with free access to water the goat first started the scratching procedure and then moved towards the water and drank (Table II).

Table II. Characteristics of a "displacement reaction" elicited by stimulation of the "drinking centre" when no water was accessible

Jan. 14th 1960			Febr. 20th 1960		
Stimul. strength mA	Scratch reaction	Drinking	Stimul. strength mA	Scratch reaction	Drinking
0.15	—	+	0.15	—	+
0.2	—	+	0.15	—	+
0.2	+	Blocked	0.15	—	+
0.2	+	Blocked	0.15	+	Blocked
0.15	+	Blocked	0.2	+	Blocked
0.2	+	+	0.2	+	Blocked
0.2	—	+	0.15	+	Blocked
0.2	+	Blocked			
0.2	—	+	30 min after Azocon, Draco		
0.2	—	+			
0.2	—	+	0.15	—	+
0.2	+	Blocked	0.15	—	+
0.2	+	Blocked	0.2	—	Blocked
0.2	+	Blocked	0.2	—	Blocked
0.2	+	+	0.2	—	+
0.2	—	+	0.2	—	+
			0.15	—	+
			0.2	—	Blocked

Bipolar stimulation between electrodes A and B.
Pulse width = 3 msec, Pulse frequency = 50 c.p.s.
For further details see text.

The effect on the scratch reaction of "psycholeptic" drugs was studied in four experiments. The intramuscular administration of 10-(3-dimethylaminopropyl)-1-azaphentiazinechloride (Azacon Draco, identical to Dominal, Homburg, 1 mg/kg b. wt.) caused a complete disappearance of the scratch reaction. The drug, however, was not seen to alter the stimulus threshold for a positive drinking response (Table II). Similar studies were also made after the intravenous administration of acepromazinemaleate (Plegicil, Pharmacia, 0.8 mg/kg b. wt.). This drug inhibited the scratch reaction but in addition markedly raised the stimulus threshold for drinking. Acepromazinemaleate also changed the behaviour of the goat in other respects during stimulation.

D. Influences of variations in pulse width and frequency on the stimulus threshold for drinking

At certain sites of electrical stimulation, changes of the stimulus parameters may alter or even reverse autonomic effects of stimulation. A frequency-conditioned reversal of blood pressure effects has been observed (KAADA 1951)

Table III. Stimulus thresholds for a positive drinking response at different parameters of stimulation applied to the "drinking centre"

	I mA	T msec	F c.p.s.	I × T × F
Jan 13th 1960	0.07	3	100	21
A → B	0.15	3	50	22.5
	0.3	3	20	18
	0.7	3	10	21
	0.07	6	50	21
	0.3	1	50	15
May 11th 1960	0.15	3	100	45
B → 0	0.3	3	50	45
	1.6	3	10	48
	0.15	6	50	45
	0.6	1	50	30

The drinking response was considered positive if the goat on repeated stimulations started to drink water at 12°C within 10 sec from the beginning of stimulation.
A → B = Bipolar stimulation between electrodes A and B.
B → 0 = Unipolar stimulation via electrode B.

and a high stimulus frequency may cause a considerable rise in arterial pressure at sites where a low frequency stimulation of the same strength does not have any circulatory effect at all (Hess 1949). For this reason it seemed to be of interest to determine the stimulus threshold for positive drinking response when stimulating with different pulse widths and different pulse frequencies.

Drinking could be induced by stimulation at a frequency as low as 10 c. p. s., whereas stimulation at a frequency of 5 c. p. s. did not induce drinking even when the stimulus strength was raised to 4 mA at a pulse width of 3 msec. As can be seen in Table III, the product of stimulus strength, pulse width and frequency (I × T × F) was relatively constant for the stimulus threshold for drinking at all parameters tested.

E. Prolonged stimulations of the "drinking centre" performed during balance studies

Unipolar stimulation via electrodes A and B apparently caused a release of neurohypophysial hormone(s) as judged from the fact that it caused milk ejection and a temporary inhibition of an established water diuresis. Further, as stimulations slightly lateral to the "drinking area" may be seen to elicit an urge to lick block salt (Andersson *et al.* 1958) it seemed to be of interest to study whether prolonged, strong, unipolar stimulations, applied via all three electrodes would affect the milk yield or the electrolyte metabolism. Unipolar stimulations (0.6 mA, 3 msec, 50 c. p. s.) were applied via the three electrodes during 5 min periods every second hour from 10.00 to 24.00.

Such experiments were made on two occasions. No other stimulations were made for 4 days before and 4 days after each experiment. The goat was kept in balance during these two 9 day periods and was not allowed to drink during the stimulations. These prolonged, strong stimulations of the "drinking area" were not observed to cause any significant change in the urinary electrolyte excretion or to alter the milk yield. Determinations of plasma chloride, sodium and potassium did not reveal any changes caused by the stimulations, whereas a 15 % rise in the blood sugar level was seen after the prolonged stimulations. The following day the blood sugar level had returned to normal.

Discussion

The results of the present study are partly a confirmation of earlier, unpublished observations made in acute experiments. The results reported here are considerably more reliable since it was possible to repeat each experiment on different occasions under standardized conditions thanks to the fact that the response to a certain stimulus remained remarkably constant during the entire period of study.

In view of the experience gained in several acute stimulation experiments with other goats it was not surprising to find that the stimulus threshold for drinking was temporarily increased when the animal was placed in an unfamiliar environment. The drinking response under such circumstances was facilitated by repeated stimulations, seemingly due to the fact that the animal gradually became adapted to the new external conditions. To avoid the interference of emotional factors, the present study was almost exclusively carried out with the goat placed in her normal environment and exposed to as few disturbing impressions as possible.

It was previously shown (Andersson and Wyrwicka 1957) that it is possible to elicit a drinking motor conditioned reaction by electrical stimulation of the hypothalamic "drinking area", and from this observation it was assumed that the "drinking area" in terms of higher nervous activity may be considered the origin of an unconditioned drinking reflex. The animal used in this study learned to overcome a simple obstacle in order to gain access to the drinking water with only few repeated stimulations. Stimulations of the "drinking area" may therefore not only elicit a drinking conditioned reaction but may also cause a motivated action obviously based on the urge to drink.

On the basis of the present study no definite conclusions may be drawn concerning the way in which the hypothalamic "drinking centre" normally functions. However, the duration of drinking persisting after stimulation and the observed affect of hydration may give some hints in this direction. In previous acute stimulation experiments it was found that drinking persisted only for short periods of time after stimulation. Generally the animals were seen to stop drinking almost immediately after cessation of stimulation (An-

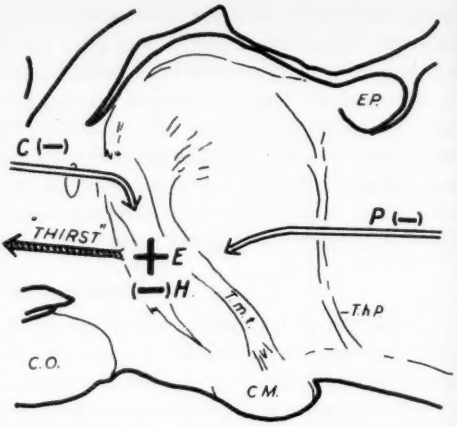

Fig. 4. A tentative explanation of the action of factors influencing the response of the "drinking centre" to electrical stimulation.
E = Electrical stimulation activating the "drinking centre".
H = Changes in the internal environment (hydration) acting directly on the "centre" by decreasing its reactivity to electrical stimulation.
C = Central (amphetamine) and P = peripheral (*i.e.* cold water) inhibitory factors acting reflexly on the "drinking centre", inhibiting the after-effect of electrical stimulation.
C. O. = Optic chiasma.
C. M. = Mammillary body.
Ep. = Epiphysis.
T. h. p. = Habenulo-peduncular tract.
T. m. t. = Mammillo-thalamic tract.

DERSSON and MCCANN 1955 a). These stimulations were, however, in most cases of short duration. In the present animal it was possible by prolonged stimulation to induce drinking which persisted for considerable time after stimulation. Under certain conditions drinking was seen to start even after the actual period of stimulation (Fig. 3 D). The similar phenomenon has recently been observed in the pigeon (ANDERSSON *et al.* 1960). A possible explanation is that the stimulated part of the brain stem may remain activated for some time after the end of stimulation. Recordings of the electrical activity from the "drinking area" just after stimulation gave some inconclusive evidence that an after-discharge may be the cause of the after-effect. Two factors were definitely shown to abolish the after-effect; the drinking of cold water and the injection of amphetamine before stimulation. The after-effect was in addition gradually reduced on repeated stimulation and was very short during hydration. In the latter case the stimulus threshold was increased but it was not significantly altered by the injection of amphetamine. This drug was shown to inhibit the drinking normally occurring after the intravenous injection of hypertonic saline in the dog. The inhibitory effect was at least in part exerted from the prefrontal areas of the brain (ANDERSSON and LARSSON 1956). The above mentioned findings may be tentatively explained by the assumption that peripheral (cold water) and central (amphetamine) inhibitory factors acting reflexly on a hypothalamic "drinking centre" block the after-discharge evoked by electrical stimulation of this "centre". Reflex inhibitory factors, however, do not seem to be forceful enough to significantly inhibit the activity of the "centre" induced during the very period of electrical stimulation. Certain changes in the internal environment (hydration) seem to be more efficient in the latter respect (Fig. 4).

Atropine given in doses which most likely inhibited the salivary secretion in the goat did not lower the stimulus threshold for drinking. This observation speaks against the "dry mouth" theory (CANNON 1918) as an explanation of the sensation of thirst.

Under the influence of a powerful urge, but at the same time prevented from expressing this urge in the appropriate way, an animal may indulge in quite "irrelevant" activity (TINBERGEN 1940). TINBERGEN and VAN IERSEL (1947) have introduced the term "displacement reactions" for these activities, which generally are characteristic for each species and may be looked upon as ways to release tensions appearing during conflict situations. TINBERGEN (1940) states that scratch movements, as seen in the chimpanzee and the orang-outang, are fairly common "displacement reactions" in mammals. The scratch reaction observed in the goat can be classified as a typical "displacement reaction". It appeared only when the animal was unable to remove the lid over the water container and was often seen in combination with other signs of distress. Other explanations to the scratch reaction seem unlikely. There does not seem to be any reason to assume that hypothalamic stimulation would cause itching located to the left side of the abdomen, especially since the goat did not exhibit the scratch reaction under the influence of a "psycholeptic" drug which in itself did not increase the stimulus threshold for drinking (Table II). The "displacement reaction" may thus be regarded as additional evidence that stimulation of the hypothalamic "drinking area" elicits a strong urge to drink.

The parameters of stimulation do not seem to be important for obtaining a drinking response to hypothalamic stimulation. If the frequency was 10 c. p. s. or higher, drinking was obtained as soon as the product of stimulus strength, pulse width and frequency reached a critical value.

Since prolonged strong stimulations of the "drinking area" were not seen to change the electrolyte metabolism of the goat or significantly increase the milk yield, the present study has produced no evidence that the stimulated part of the hypothalamus exerts any influence on the anterior pituitary or the adrenal cortex.

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Alveolo-Arterial Gas Exchange at Rest and During Work at Different O_2 Tensions

By

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Abstract

ASMUSSEN, E. and M. NIELSEN. *Alveolo-arterial gas exchange at rest and during work at different O_2 tensions.* Acta physiol. scand. 1960. *50.* 153—166. — The mean alveolar and the arterial gas tensions in healthy young male subjects have been determined at rest and during muscular exercise of varying intensities up to maximal work. Determinations were made while the subjects were breathing air containing 33 to 50 per cent, 21 per cent and 12 per cent oxygen. From the experimental data the alveolo-arterial oxygen tension differences and the arterial oxygen deficits (*i. e.* the difference between the oxygen content of blood in full equilibrium with the mean alveolar air and the actual oxygen content of peripheral arterial blood) were calculated. Under the assumption that the BOHR integration procedure can be used for determining the mean pulmonary capillary P_{O_2} in the human lung it can be concluded that the arterial oxygen deficit when breathing normal air both at rest and during maximal work is caused by true venous admixture (anatomical shunts) and the effect of uneven distribution in the lungs, and it is found that about 80 per cent is due to the distribution effect and the remaining 20 per cent to the true venous admixture. Further, the effect of the distribution factor at low oxygen pressures increases manifold as compared with the effect at normal O_2 pressure. The large arterial O_2 deficit found in the low O_2 experiments (in ml per min equal to 62 and 36 per cent of the total O_2 uptake at rest, respectively during maximal work) can, therefore, be explained by the true venous admixture and the greatly increased effect of the distribution factor. According to this view it is not possible to determine the lung diffusion capacity for O_2 on the basis of the alveolo-end pulmonary capillary

O_2 gradient by the procedure of RILEY and COURNAND (which assumes a constant effect of the distribution factor at high and low oxygen tension) or by the procedure of BARTELS *et al.* (which does not take the distribution factor into account).

The early direct determinations of arterial oxygen tension by KROGH and KROGH (1910) revealed the existence of an alveo-arterial O_2 tension difference (A-a P_{O_2} difference). This finding has been confirmed by numerous investigators (for references see FORSTER 1957 and ROSSIER, BÜHLMANN and WIESINGER 1958). The causes for the A-a P_{O_2} difference have been analyzed among others by BERGGREN (1942), LILIENTHAL *et al.* (1946), RILEY and COURNAND (1951) and BARTELS *et al.* (1955 a—c).

In earlier experiments concerned with the regulation of respiration data were obtained from which the A-a P_{O_2} difference could be calculated. These data have been supplemented by values from special experiments in the hope that this material, comprising results from experiments in rest and work at high and low oxygen tensions, might contribute to the analysis of the causes for the A-a P_{O_2} difference.

Methods

Arterial gas tensions were determined by a bubble equilibration method described earlier (ASMUSSEN and NIELSEN 1958). The arterial blood was drawn from the brachial artery through an indwelling arterial needle, which in the rest experiments was placed in the artery 5 to 10 min and in the work experiments 3 to 4 min before the sampling of blood began. The sampling was extended over a period of 3 to 6 minutes. The blood was collected under icecold paraffin oil and immediately transferred to cooled syringes. The equilibration of the introduced gas bubble took place in the same syringes after rewarming in water to 37° C.

At high oxygen tensions special precautions were taken to eliminate loss of oxygen from the blood sample to the surroundings (paraffin oil, rinsing fluid etc.). For this purpose these fluids were equilibrated with air mixtures of the same partial gas pressures as those expected in the blood. In order to correct for the fall of oxygen tension due to the oxygen consumption of the blood during the bubble equilibration, analyses of blood equilibrated with known gas mixtures of approximately the same partial pressures as the arterial tensions were performed in all series of determinations parallel to the analyses of the arterial blood. The corrections determined in this way at normal and low oxygen tensions were 0 to + 2 mm Hg for P_{CO_2} and — 0.5 to + 1 mm Hg for P_{O_2}. At the high oxygen tensions (200—300 mm Hg) the correction for P_{CO_2} was the same but the correction for P_{O_2} averaged + 22 to + 27 mm Hg. The main reason for the large correction of the P_{O_2} at the high oxygen tensions is that the same fall in oxygen content due to the oxygen consumption of the blood causes a much larger fall in tension than at a lower P_{O_2}. Further, we have found in as yet unpublished experiments that the oxygen consumption of blood increases with the oxygen tension. In the low oxygen experiments the arterial P_{O_2} was determined also from the oxygen content and the pH of the arterial blood by use of a standard oxygen dissociation curve. As seen in Table I the two methods gave identical results. The measured gas tensions were corrected to the subjects' rectal temperature as measured immediately after the blood sampling. At low and normal oxygen tensions the temperature corrections were those mentioned earlier (ASMUSSEN and NIELSEN 1958). At high oxygen

Table I

Rest

O_2 in inspir. air.		alveolar I P_{CO_2} end tidal mmHg	alveolar II P_{O_2} end tidal mmHg	alveolar III P_{O_2} mean mmHg	arterial IV P_{CO_2} direct mmHg	arterial V P_{O_2} direct mmHg	arterial VI P_{O_2} from Hb O_2% mmHg[1]	arterial VII Hb O_2 %	VIII A–a P_{O_2} diff. (III—V) mmHg
12%	mean ..	*32.2*	*42.6*	*42.8*	*33.6*	*35.8*	*35.9*	*71.3*	*7.1*
	S. E. ±	0.51	0.82	0.35	0.85	[2]0.79	0.24	0.38	[2]0.97
	number	10	10	12	12	12	12	12	12
21%	mean ..	*36.7*	*102.7*	*104.6*	*36.9*	*87.6*			*17.0*
	S. E. ±	0.50	0.84	0.85	0.56	[2]1.04			[2]0.97
	number	18	18	18	18	18			18
50%	mean ..	*38.8*	*296.2*	*294.6*	*38.2*	*274.1*			*19.7*
	S. E. ±	0.14	3.35	3.20	0.13	[2]2.05			[2]2.71
	number	13	13	11	13	13			11

[1] corrected to observed pH and temp.
[2] including S. E. of correction.

pressures the P_{O_2} values were corrected under the assumption that only physically dissolved oxygen changed with temperature.

The mean alveolar oxygen tension was calculated from the arterial P_{CO_2} and the gas tensions of inspired and expired air (RILEY *et al.* 1946) as:
mean alveolar P_{O_2} = inspired P_{O_2}-arterial P_{CO_2}/expired P_{CO_2} (inspired P_{O_2}-expired P_{O_2}) assuming that the physiological dead space for CO_2 equals the physiological dead space for O_2 (ASMUSSEN and NIELSEN 1956). The alveolar P_{O_2} calculated in this way is identical to the "effective" alveolar P_{O_2} as originally calculated by RILEY and COURNAND (1949) from arterial P_{CO_2} and the RQ of the expired air using the more complicated "alveolar air equation" of FENN, RAHN and OTIS (1946). In the rest experiments the alveolar gas tensions were also determined from samples of end-tidal air taken by the automatic device described by NIELSEN and SMITH (1951). There was good agreement between the results obtained by the two methods. The average P_{CO_2} values differed from — 0.6 to + 1.4 mm Hg and the P_{O_2} values from — 1.6 to + 1.9 mm Hg.

The expired air was collected in DOUGLAS bags. In the experiments in which the inspired air was not atmospheric air the subject breathed from another DOUGLAS bag that was continuously refilled from a cylinder containing the air mixture. The sampling of expired air and alveolar air took place simultaneously with the sampling of arterial blood.

The subjects were young male students, not especially trained for muscular exercise, who were studied once a week. Each person served as a subject 2 to about 10 times. For the rest experiments they came fasting to the laboratory in the morning and lay down for half an hour before the experiments. All the rest experiments were performed with the subjects in a lying position. The work experiments were performed on a chair cycle ergometer. The gas and blood sampling took place after a steady state had been reached.

Table II

Heavy work at different O_2-tensions. O_2-uptakes 2.6—3.3 l/min.

O_2 in inspir. air	number	mean alveolar P_{O_2} calculated mmHg	arterial P_{CO_2} dir. mmHg	arterial P_{O_2} dir. mmHg	A—a P_{O_2} diff. mmHg	S. E.[2] mmHg
12%	4	58.6	[1]29.8	[1]38.4	20.2	—
21%	9	111.7	36.7	87.6	24.1	± 1.33
33%	13	203.2	38.2	179.0	24.2	± 2.81
50%	14	307.1	38.9	279.3	27.4	± 3.13

[1] from arterial Hb O_2%, pH and CO_2-content, corrected to observed temp.
[2] including S. E. of correction.

Results

Table I contains alveolar and arterial gas pressures from experiments at rest with different oxygen percentages in the inspired air. The last column contains the A-a P_{O_2} difference determined from the arterial P_{O_2} and the mean alveolar P_{O_2} as calculated from the formula on p. 155. In earlier studies (cf. Table IV in BARTELS *et al.* 1955 b) A-a P_{O_2} differences at rest during air breathing have been found to be from 7 to 10 mm Hg. At high oxygen pressure only few determinations of the A-a P_{O_2} difference have been performed earlier. In rest experiments with 100 per cent oxygen in the inspired air BERGGREN (1942) found an average A-a P_{O_2} difference of 11 mm Hg, and with 50 per cent oxygen BARTELS *et al.* (1955 c) found 14.9 mm Hg. At low oxygen pressure, LILIENTHAL *et al.* (1946) and BARTELS and RODEWALD (1953) found an average A-a P_{O_2} difference of 9 and 4 to 5 mm Hg respectively. Table II

Table III

A—a P_{O_2} difference at different levels of oxygen uptake

O_2-uptake l/min.	number	mean alv. P_{O_2} mmHg	arterial P_{CO_2} mmHg	arterial P_{O_2} mmHg	A—a P_{O_2} diff. mmHg	S. E.[1]
0.29	18	102.7	36.9	87.6	17.0	± 0.97
1.49	10	105.8	40.1	88.7	17.1	± 1.04
1.82	10	104.7	39.1	84.7	20.0	± 1.61
2.89	15	110.0	37.3	83.8	26.2	± 1.16
3.30	9	111.7	36.7	87.6	24.1	± 1.33
[2]3.49	13	123.3	29.1	87.9	35.0	± 1.15

[1] including standard error of correction.
[2] measured during final spurt to exhaustion.

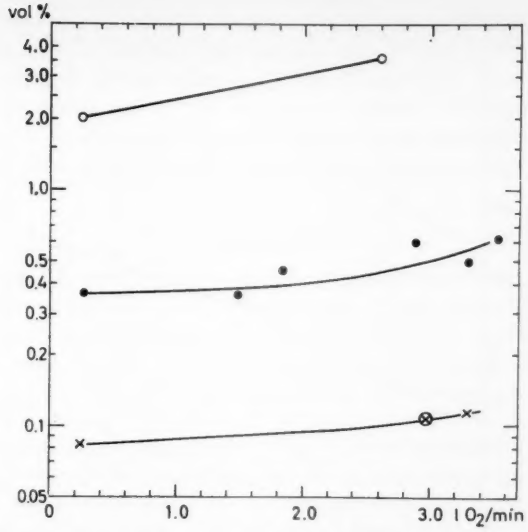

Fig. 1. Arterial oxygen deficit in vol% (log. scale) in relation to oxygen consumption. Inspired air 50 % O_2 (×), 33 % O_2 (⊗), 21 % O_2 (●) and 12 % O_2 (○).

shows results from experiments during heavy work at oxygen percentages from 12 to 50 per cent; Table III shows the results from work experiments in air with increasing work loads. The A-a P_{O_2} difference is practically the same in light work as during rest but it increases at the higher work intensities. The most severe work was performed as a final spurt to exhaustion (pulse rate 200 to 240) lasting 1 to 2 min, after a warming-up period with heavy work of about 10 min duration. Table III shows that the increased A-a P_{O_2} difference found was due to the increased alveolar P_{O_2} and not to a fall in arterial P_{O_2}. In exhaustive work, HOLMGREN and LINDERHOLM (1958) found a decrease in arterial P_{O_2}. This discrepancy may be due to a higher O_2 consumption reached by their subjects who rode an ordinary bicycle ergometer, whereas the present experiments were performed with a chair ergometer. BARTELS *et al.* (1955 b, Table IV) have summarized the A-a P_{O_2} differences from experiments with light and moderate work in air of earlier investigators. They vary from 7 to 19.6 mm Hg and exceeded in most cases the corresponding values at rest. During work in hypoxia LILIENTHAL *et al.* (1946) found an average A-a P_{O_2} difference of 16.9 mm Hg, *i. e.* the same as in their air experiments. During moderate work in 50 per cent oxygen BARTELS *et al.* (1955 c) found an A-a P_{O_2} difference of 25.8 mm Hg as compared to 14.9 mm Hg during work in atmospheric air.

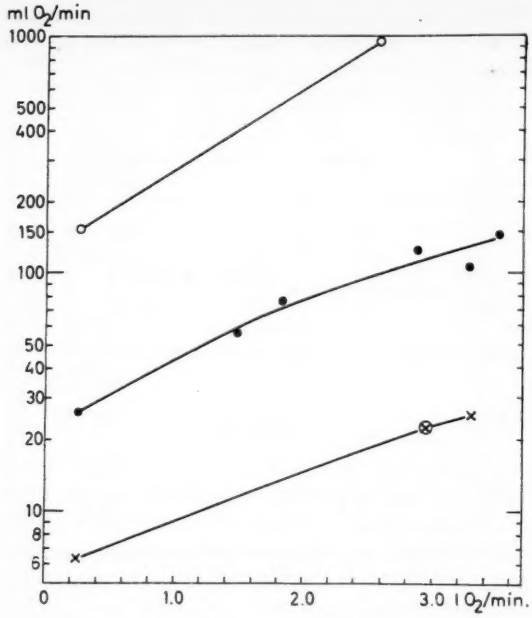

Fig. 2. Arterial oxygen deficit in ml per minute (log. scale) in relation to oxygen consumption. Signatures as in Fig. 1.

From the A-a P_{O_2} differences in Table I—III, the "arterial oxygen deficits" were calculated, *i. e.* the difference between the oxygen content of arterial blood, if this had been in full equilibrium with the mean alveolar air, and the actual O_2 content of the peripheral arterial blood. For the calculations we have used the mean alveolar and the arterial P_{O_2} values (Table I—III), the O_2-dissociation line chart of SEVERINGHAUS from *Handbook of Respiration* (1958) for bound oxygen, and an oxygen solubility coefficient of 0.003 ml O_2 per mm P_{O_2} for free oxygen. The oxygen capacity of the blood was assumed to be 22 vol%. According to this calculation (Fig. 1) the oxygen deficit in vol% is small at high oxygen pressures, 4 to 5 times larger in the air experiments and 25 to 35 times larger at low oxygen. The deficit increases with increasing oxygen uptake.

From the arterial deficit in vol% the arterial deficit in ml O_2 per minute can be derived through multiplication by the cardiac output expressed in deciliters per min. Values for the cardiac output at rest and during work in air, low oxygen and high oxygen were taken from previously published data (ASMUSSEN and NIELSEN 1952 and 1955). The arterial oxygen deficit

in ml per minute increased with work and was the larger the lower the oxygen pressure (Fig. 2). In rest breathing 50 per cent, 21 per cent or 12 per cent oxygen the oxygen deficit corresponded to 2.4 per cent, 10.4 per cent and 62 per cent, respectively, of the total oxygen uptake. During work with an oxygen uptake of about 2.6 l per min the corresponding figures were 0.7 per cent, 3.8 per cent and 36 per cent at high, normal and low oxygen pressures.

Discussion

The reasons for the existing A-a P_{O_2} difference have been discussed by several authors (HALDANE and PRIESTLY 1935, BERGGREN 1942, LILIENTHAL *et al.* 1946, RILEY and COURNAND 1951. BARTELS 1955, a. o.). They are usually ascribed to three factors: 1) Venous admixture, 2) uneven distribution of ventilation and circulation in the lungs and 3) failing diffusion equilibrium between alveolar air and blood.

The venous admixture component is due to venous blood entering the pulmonary veins (*e. g.* from the bronchial veins) or the left heart (*e. g.* from the Thebesian veins). The effect of this factor on the arterial oxygen deficit depends on the amount and the oxygen deficit of the admixed venous blood. Because of the shape of the O_2-dissociation curve the effect on the A-a P_{O_2} difference will be relatively large at high arterial oxygen tensions and almost negligible at low arterial oxygen tensions.

Uneven distribution of the ratio ventilation: circulation in the lungs will influence both alveolar and arterial gas tensions. By calculating the alveolar P_{O_2} on the basis of *arterial* P_{CO_2} (see p. 155), (which is only to a negligible degree influenced by uneven ventilation and circulation because of the form of the CO_2 dissociation curve), it may be assumed that the best possible value for a mean alveolar P_{O_2} is obtained. The only assumption for this calculation (see p. 155) is that the physiological dead space for CO_2 and O_2 are equal, an assumption which seems justifiable since, during rest, the dead spaces calculated from the expired alveolar gas tensions are equal for the two gases (NIELSEN 1936 p. 105).

While an uneven distribution of ventilation and circulation in different parts of the lung has only a negligible effect on the arterial P_{CO_2} it may cause a distinct diminution of the arterial P_{O_2}. This is due to the fact that the O_2 saturation of the blood will be influenced relatively more in the hypoventilated than in the hyperventilated parts of the lung. This effect on the O_2 saturation is most pronounced at O_2 tensions at which the slope of the O_2 dissociation curve changes the most. On the other hand a certain desaturation of the blood will have the greater effect on arterial P_{O_2} the closer the blood is to 100 per cent O_2 saturation. RAHN (1949), FARHI and RAHN (1955) and BRISCOE (1959) have attempted to evaluate these effects of different distribution of ventilation and circulation in the lung on the size of the A-a P_{O_2} gradient.

The third component of the A-a P_{O_2} gradient, the diffusion factor, is determined by the total resistance to the transfer of O_2 from the alveolar air to the hemoglobin molecule. The total resistance comprises the resistance of the pulmonary membrane, the intracapillary resistance to diffusion, and the delay due to the reaction of O_2 with hemoglobin.

a. True venous admixture component. The influence of the venous admixture can be determined in experiments with breathing of high oxygen (BERGGREN (1942), BARTELS *et al.* (1953, 1955 a—c)). In the experiments presented here breathing of 50 per cent oxygen was used at rest and during maximal work. With 50 per cent oxygen in the inspired air the alveolar hyperoxia is large enough to eliminate the diffusion and the distribution factors. This is seen from the fact that the results from experiments with only 33 per cent oxygen in the inspired air also fall in line with the results from the 50 per cent oxygen experiments (see Fig. 1 and 2).

The venous admixture, both for rest and work conditions, is commonly expressed as a percentage of the cardiac output. In our experiments, however, (Fig. 1 and 2) the effect of the venous admixture has been expressed only as arterial oxygen deficit, because the a-v O_2 difference of the admixed venous blood is unknown and, at least for the blood from the Thebesian veins, probably differs much from the resting a-v O_2 difference of the mixed venous blood. Further, there is no reason to assume that the a-v O_2 difference of the nutritive blood from the bronchi and from the heart increases during muscular exercise, whereas the a-v O_2 difference of the mixed venous blood increases up to about four-fold.

From Fig. 2 it is seen that the arterial oxygen deficit due to the venous admixture increases from about 6 ml per min at rest to about 25 ml per min during maximal work. This large increase in arterial oxygen deficit makes it probable that blood from the bronchi, whose oxygen demand hardly increases during work, plays only a minor part in the venous admixture. Therefore, provided that no other direct shunts exist, the main part of the venous admixture probably comes from the heart, whose oxygen consumption increases with increasing intensity of work.

The true venous admixture component determined in the hyperoxia experiments must be assumed to be practically the same in the experiments with normal air breathing and in the experiments with low oxygen. Both at rest and during work it amounted to about 20 to 25 per cent of the total arterial oxygen deficit in the air experiments and to only 3 to 4 per cent of the total arterial oxygen deficit in the low oxygen experiments (Fig. 1). The remaining part of the arterial oxygen deficit while breathing normal air or low oxygen is then due to the distribution factor and the diffusion factor.

b. The distribution and the diffusion components. LILIENTHAL *et al.* (1946), RILEY and COURNAND (1951) and RILEY, COURNAND and DONALD (1951) have attempted to isolate the diffusion factor from the two other components of the

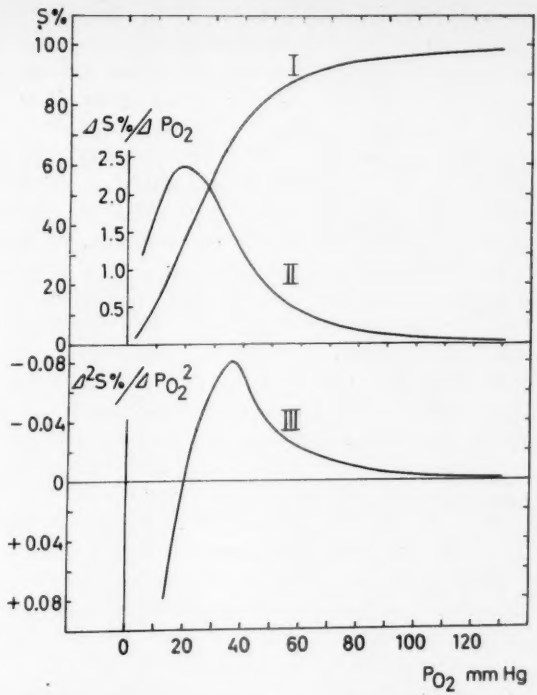

Fig. 3. I. Standard HbO_2-dissociation curve at pH 7.4 and 37° C (S% in relation to P_{O_2}). II. Slope of curve I ($\Delta S\%/\Delta P_{O_2}$ in relation to P_{O_2}). III. Slope of curve II ($\Delta^2 S\%/\Delta P_{O_2}^2$ in relation to P_{O_2}).

A-a P_{O_2} difference. The true venous admixture factor and the distribution factor were combined into one, called "venous admixture" or physiological shunt and expressed as a percentage of the cardiac output. The main difficulty in the analysis is the determination of the end-pulmonary capillary P_{O_2}, which subdivides the A-a P_{O_2} difference into one part due to diffusion and another due to "venous admixture". The diffusion factor, *i. e.* the alveolo-end pulmonary capillary O_2 gradient, and the "venous admixture" were determined in experiments performed at two levels of oxygenation (breathing room air and breathing about 14 per cent O_2) by a "trial and error method" under the assumption that the "venous admixture" and the lung diffusion capacity for oxygen, D_{O_2}, remained constant at both levels of oxygenation. Using the found end pulmonary capillary P_{O_2}, the lung D_{O_2} was determined by means of the BOHR integration procedure. By this method, LILIENTHAL *et al.* (1946) found that of a total A-a P_{O_2} gradient of 9 mm Hg in rest at normal air 0.2

mm Hg was due to the diffusion factor and 8.8 mm Hg to "venous admixture", whereas in low O_2, with a total A-a P_{O_2} gradient of 9.5 mm Hg, 8.9 mm Hg was due to diffusion and only 0.6 mm Hg to "venous admixture". On the basis of these figures D_{O_2} was calculated to be 21 ml/min/mm Hg.

While it may be true that no significant changes in D_{O_2} occur when passing from one to another of the two levels of oxygenation used in the "trial and error method" (from 96 to 82 per cent Hb-saturation) it can not be assumed that the effect of the "venous admixture" is the same at the two levels of oxygenation: The mixing of two portions of blood coming from relatively hypoventilated and hyperventilated parts of the lung must cause a much larger decrease of arterial saturation at the low than at the high level of oxygenation. This can be seen from Fig. 3 in which curve I is a standard dissociation curve (HbO_2 saturation (S%) in relation to P_{O_2}) at pH 7.4 (SEVERINGHAUS in *Handbook of Respiration*, 1958). Curve II represents the slope of this dissociation curve ($\triangle$ S%/$\triangle$ P_{O_2}) in relation to P_{O_2}, and curve III the slope of curve II, *i. e.* the change of slope of the dissociation curve ($\triangle^2$S%/$\triangle$ $P_{O_2}^2$) in relation to P_{O_2}. If two portions of blood with different P_{O_2} are mixed the S% of the mixed blood will differ from that of blood with a P_{O_2} equal to the average P_{O_2} of the two blood portions. The difference is proportional to the ordinate in curve III, as is the fraction of the "venous admixture" due to uneven distribution — provided the pH of the blood were constant throughout the lung. As seen in Fig. 3 this ordinate is much larger at a P_{O_2} corresponding to the low level of oxygenation than at a P_{O_2} corresponding to the high level of oxygenation used by LILIENTHAL *et al.* In reality blood coming from hypoventilated alveoli will have a lower pH than blood coming from hyperventilated alveoli. This difference in pH will further increase the lowering effect of uneven distribution on the O_2 saturation of the blood and this effect will, likewise, be larger at the low than at the high level of oxygenation.

In the experiments presented here the arterial oxygen deficit during rest, breathing atmospheric air, was found to be 0.36 vol% (see Fig. 1). In this condition (rest, breathing air) the alveolo-end pulmonary capillary O_2 gradient can probably be considered negligible: As pointed out by BERGGREN (1942), the lung diffusion capacity as found by the CO-method (M. KROGH 1915) is of such a magnitude that the end O_2 gradient becomes at the most a small fraction of a mm Hg. Even though the validity of the method used for arriving at this conclusion (BOHR integration procedure) may be doubted under conditions of uneven distribution of ventilation and circulation in the lungs, we shall assume it to be justified, and the following discussion will be based on the concept that no or practically no diffusion end gradient is present at rest breathing normal atmospheric air. Therefore, in our rest experiments the arterial oxygen deficit of 0.36 vol% must be due only to true venous admixture (0.08 vol%) and to the distribution factor (0.28 vol%). In the low O_2 experiments the arterial O_2 deficit is about 2.0 vol%. Assuming the true

venous admixture to be the same as in the air experiments the remaining part of the arterial oxygen deficit in the low oxygen experiments is about 1.92 vol%, which is less than expected if the distribution effect increased as estimated from curve III in Fig. 3. Consequently even at this low level of oxygenation there is no reason to assume that any essential part of the A-a P_{O_2} gradient is due to a failing diffusion equilibrium. That the arterial O_2 deficit in the low oxygen experiments (S% = 71.3) is less than expected might be due to regulatory changes diminishing the difference in P_{O_2} between blood from hypo- and hyperventilated parts of the lung.

The arterial oxygen deficit found in our rest experiments in air is somewhat higher than found by other authors. We have calculated the O_2-deficits from the corresponding experiments of LILIENTHAL *et al.* (1946) and also from the large material of FILLEY *et al.* (1954) and found it to be 0.20 vol% and 0.27 vol% (O_2 capacity assumed to be 22 vol%) respectively as compared to 0.37 vol% in our experiments. Even with these smaller values of the arterial O_2 deficit, and assuming the same true venous admixture as we have found, the increase in the distribution effect occurring at the lower values of P_{O_2} is larger than necessary to explain the whole remaining part of the arterial O_2 deficit, both at the low level of oxygenation used by LILIENTHAL *et al.* (S% about 70) and at the somewhat higher level of oxygenation (S% about 82) later proposed by RILEY and COURNAND in their "trial and error method".

From the above discussion, it appears that practically the whole A-a P_{O_2} gradient in rest, both when breathing normal air and when breathing 12 to 14 per cent O_2, can be explained as being due to venous admixture and to uneven distribution. Especially at low O_2 tensions uneven distribution constitutes, by far, the predominant part. No or practically no gradient due to failing diffusion will consequently exist in these conditions and it will not be possible to determine the lung diffusion constant for $O_2(D_{O_2})$ by the "trial and error method". The values for D_{O_2} found under the assumption of a constant "venous admixture" will, therefore, be much too small.

BARTELS *et al.* (1955 a) determined the true venous admixture in experiments with hyperoxia (35 per cent O_2 in the inspired air) and calculated D_{O_2} from experiments in air and in low oxygen under the assumption that the A-a P_{O_2} gradient was due only to the true venous admixture and to a failing diffusion equilibrium. This assumption was based on the observation that "venous admixture" was of the same size when breathing air and when breathing 35 per cent O_2, an observation which was not confirmed in later experiments by BARTELS *et al.* (1955 c). — Since the distribution factor is not taken into account values of the lung diffusion capacity determined by this procedure will be much too low.

c. The size of the lung diffusion capacity. We do not consider it possible to calculate the exact D_{O_2} values from an analysis of the A-a P_{O_2} gradient because, among other reasons, there may be no or practically no O_2 end gradient

even in the low O_2 experiments. If, however, an end O_2 gradient of 1 mm Hg is assumed the D_{O_2} in our low oxygen experiments would be 54 ml/min/mm Hg as compared to 27 ml/min/mm Hg if the end-gradient, as in the procedure of RILEY and COURNAND, was practically equal to the whole A-a P_{O_2} difference of about 7 mm Hg. For comparison with the above mentioned value for D_{O_2}, 54 ml/min/mm Hg, it can be mentioned that FORSTER (1957), using a formula first reported by KRUHØFFER (1954), corrected the diffusion capacity for CO (D_{CO}) in rest (about 25 ml/min/mm Hg) for the delay due to intracellular diffusion and the reaction of CO with Hb, converted it to D_{O_2} by multiplication with 1.23, and found it to be 63 ml/min/mm Hg. This value (the isolated pulmonary membrane diffusion capacity for oxygen) was used by FORSTER in combination with the D_{O_2} value of 21 ml/min/mm Hg (depending both on the resistance of the pulmonary membrane and on the intracapillary resistance) found by RILEY and *coworkers*, for estimating the intracapillary resistance for oxygen. In that way he found that this latter also, like the intracapillary resistance to CO, constituted about 50 per cent of the total resistance from the alveolar air to the Hb molecule. However as mentioned above, there may, even in the low O_2 experiments be practically no alveolo-end pulmonary capillary O_2 gradient. A D_{O_2} value of 54 ml/min/mm Hg, calculated on the basis of an end-gradient of 1 mm Hg, must therefore be considered as a minimum value. Consequently FORSTER, using a D_{O_2} value of 21 ml/min/mm Hg, must have overestimated the intracapillary resistance to O_2.

During work in atmospheric air (O_2 consumption 3.3 l per min) and assuming an alveolo-end pulmonary capillary O_2 gradient of 1 mm Hg we find that the D_{O_2} is 58 ml/min/mm Hg. In heavy work of the same intensity BØJE (1934) found an average D_{CO} value of 42 ml/min/mm Hg which converted to D_{O_2} is 52 ml/min/mm Hg. These latter values are determined not alone by the membrane resistance but also by the intracapillary resistance to CO, which according to ROUGHTON and FORSTER (1957) is of about the same magnitude as the former. The true diffusion capacity of the pulmonary membrane must, therefore, be much larger than 52 ml/min/mm Hg, and, as the present experiments indicate that the intracapillary resistance to O_2 is only slight, the D_{O_2} value of 58 ml/min/mm Hg must probably be a minimum value. The assumed end gradient of 1 mm Hg is, therefore, probably a maximum value. It follows that, in maximal work in atmospheric air, the arterial oxygen deficit, as in rest, is due almost exclusively to true venous admixture and to uneven distribution.

At an O_2 uptake of 2.6 l/min in atmospheric air the arterial O_2 deficit is 0.46 vol% (0.1 vol% due to true venous admixture and 0.36 vol% due to the distribution factor, cf. Fig. 1). In *low* O_2 at the same work intensity, the arterial O_2 deficit is 3.6 vol%, of which 0.1 vol% is due to true venous admixture. The remaining part, 3.5 vol% can (cf. Fig. 3) be explained as in

rest, *i. e.* by the increased effect of the distribution factor at low P_{O_2}. There is, therefore, even during maximal work at low O_2, no or at least only a small alveolo-end pulmonary capillary O_2 gradient. Assuming a small end O_2 gradient, *e. g.* about 2 mm Hg, the D_{O_2} would then be about 140 ml/min/mm Hg as compared to 84 ml/min/mm Hg if the end O_2 gradient as in RILEY *et al's.* procedure was practically equal to the whole A-a P_{O_2} difference of about 20 mm Hg.

The preceding discussion of the alveolar gas exchange has as mentioned before (p. 162) been based on the assumption that the BOHR integration procedure can be used for determining the mean pulmonary capillary P_{O_2}. If, however, this assumption is not correct it is not impossible that a failing diffusion equilibrium exists in the relatively hypoventilated parts of the lung — an assumption that as far as we know, has not been put forward. In that case a much larger part of the arterial oxygen deficit at low oxygen would be explained by the diffusion factor and it would not be possible to analyze the arterial oxygen deficit with respect to the parts played by diffusion and uneven distribution in the way here attempted.

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On the Reflex Effects from the Knee Joint of the Cat

By

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Abstract

Ekholm, J., G. Eklund and S. Skoglund, *On the reflex effects from the knee joint of the cat*. Acta physiol. scand. 1960 *50*. 167—174. — The reflex effects on extensors and flexors of the knee joint have been studied in response to various stimulations of the knee joint capsule and the medial collateral ligament. The joint receptors were activated by increasing the intra-articular pressure or by pinching the anterior aspect of the knee joint capsule. This stimulation caused an inhibition of the knee extensors and a facilitation of the knee flexors in both the decerebrated and decerebrated and spinalized preparation. Activation of the receptors in the medial collateral ligament was likewise found to produce a flexor facilitation in the decerebrated and spinalized animal, effects that sometimes could be produced even in the decerebrated animal.

The anatomy and physiology of joint receptors have been the subject of much experimental research during the last decade. The reflex effects that can be set up from joint afferents have also been investigated to some extent, but the results reported are contradictory. Gardner (1950) studied the reflex effects of electrical stimulation of the nerves to the knee joint in the cat, and got very variable results in the decerebrated animal. In the spinalized preparation, on the other hand, stimulation of the joint afferents always activated the flexor muscles. Since Gardner only observed the visible muscle contractions his results are difficult to evaluate.

Skoglund (1956) also studied the reflex effects set up by electrical stimulation of the nerves to the knee joint in the cat. He measured the effects as variations induced in the monosynaptic reflex response from various hindlimb nerves. He found the effects to be very variable from experiment to experiment. He suggested that the explanation for the variations seen was that electrical

excitation of the joint nerves activates fibres connected to sensory endings functioning at different positions and movements of the joint simultaneously and they are likely to have different central effects, which might cancel each other. SKOGLUND (1956) also tried adequate stimulation of the joint receptors for setting up reflex effects. Extensive denervations and extremely rigid fixation of the limb were, however, found necessary to exclude false effects from muscle receptors. This limits the possibilities to investigate the reflex effects of joint movements (cf. SKOGLUND 1956).

In a recent publication STENER (1959) has reported a method of stimulating the receptors in the medial collateral ligament of the knee rather selectively. ANDERSSON and STENER (1959), were, however, unable to produce any reflex effects by such selective stimulation of the ligamentory receptors. PETERSÉN and STENER (1959) extended this study to man and were unable to produce any reflex effects by abduction of the knee joint, which increases the tension in the medial collateral ligament and activates the ligamentory receptors. These results are quite contradictory to those presented by PALMER (1958) who reported evidence for the existence of ligamento-muscular protective reflexes.

These controversial results raise the question whether the earlier described small reflex effects set up by stimulation of joint nerves are of any physiological importance at all. The aim of the present paper was originally to study the effects of joint pain on muscular reflexes. During the investigation the paper of ANDERSSON and STENER (1959) appeared and it was decided to adopt their technique of stimulation and use it in other preparations than the decerebrated animal, which was the only one used by these authors. The effects set up were studied on the monosynaptic response from various hindlimb nerves and on electromyograms.

Methods

Twenty-six adult cats have been used. All animals were decerebrated under ether anesthesia. Some were spinalized by a section at Th 8—10 before the experiment started and some during the experiment. Two hours were allowed for the ether to be removed. The temperature of the animal was kept at 36—39° C by placing them on a heated table and warming them by lamps. All dissected nerves and roots were covered by paraffin pools at 37° C.

Monosynaptic potentials were set up from various muscle nerves and recorded either from cut ventral roots or from muscle nerves. In the experiments with monosynaptic testing all nerves in the limb were cut except the branches to the knee joint running via the saphenous nerve which was cut distally to the point where it gives off its articular branches. The iliopsoas muscle was cut in addition.

The electromyogram was recorded from the following muscles: quadriceps, biceps, semitendinosus and semimembranosus. In the experiments using electromyography the saphenous nerve was also cut distal to the point where it gives off its articular branches. Usually the sciatic nerve was cut in the popliteal fossa. The posterior nerve to the knee joint was thus sacrificed by this procedure.

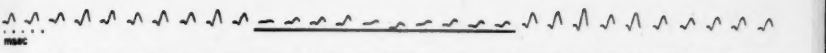

Fig. 1. Decerebrated cat. Monosynaptic test volleys set up and recorded from the cut quadriceps nerve. The iliopsoas muscle and all nerves in the limb were cut except those to the knee joint running via the saphenous nerve. The monosynaptic reflex was elicited once per second. Inhibition produced by increasing the pressure in the knee joint cavity during the time indicated by the signal line. Time in msec to the left.

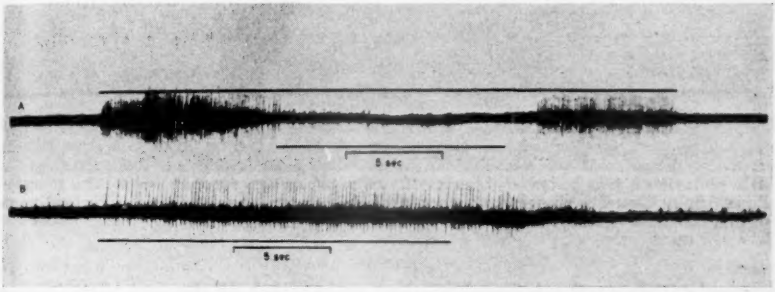

Fig. 2. Decerebrated cat. A. The electrical activity in the quadriceps muscle set up by pinching the contralateral heel (indicated by upper signal line). Inhibition produced by squeezing the ipsilateral joint capsule during the time indicated by the lower signal line. B. Same cat. The electrical activity of the biceps muscle. Signal line indicates squeezing of the ipsilateral joint capsule.

The stimuli setting up monosynaptic reflex responses were square wave shocks of 0.5 msec duration delivered through Ag—AgCl hooks. Monosynaptic potentials were led off in conventional way. The electromyogram was recorded by steel needles insulated to the tip and inserted in the muscle bellies.

The joint receptors chiefly connected to the medial nerve (see above) were activated in the following ways: In one series of experiments a cannula was inserted into the knee joint cavity from the lateral side. By means of a syringe filled with Ringer solution the pressure in the joint cavity could be altered at will. Increasing the intra-articular pressure is known to activate the joint receptors (ANDREW and DODT 1953). In another series of experiments a thread was fastened around the medial collateral ligament according to the method of STENER (1959). By loading the thread or applying traction to it by hand, the tension of the ligament is increased and the ligamentory receptors are to some extent selectively activated. In both these series of experiments the joint receptors were also activated by pinching or squeezing the joint capsule by a pair of insulated forceps. In all experiments the limb used was very rigidly fixed in holders according to STENER (1959).

Results

Increasing the intra-articular pressure which as shown by ANDREW and DODT (1953) activates the joint receptors, usually caused an inhibition of the monosynaptic reflex from quadriceps, Fig. 1. Squeezing the joint capsule on the anterior aspect of the joint had the same effect. In Fig. 2 A is seen the

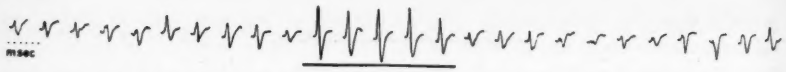

Fig. 3. Spinal cat. Monosynaptic test volleys set up and recorded from the nerve to the knee flexors (biceps-semitendinosus). Experimental situation as in Fig. 1. Volleys elicited once per sec. Facilitation of the reflex response produced by loading the string sewn around the medial collateral ligament during the time indicated by the signal line. Time in msec to the left.

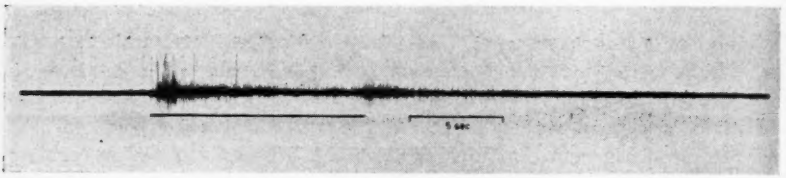

Fig. 4. Spinal cat. The electrical activity of the semimembranosus muscle set up by loading the medial collateral ligament during the time indicated by the signal line.

crossed extensor reflex in quadriceps set up by pinching the heel of the contralateral limb. This activity as shown in the figure is easily inhibited by pinching the anterior aspect of the knee joint capsule. In Fig. 2 B the same way of activating the knee joint receptors is seen to set up activity in the biceps femoris. The effects shown in Fig. 1 and 2 were as easily obtained in the decerebrated as in the decerebrated and spinalized preparation.

In the experiments where the receptors of the medial collateral ligament were activated by rapidly increasing the tension in the ligament (cf. STENER 1959) the flexors and extensors of the knee joint could be influenced. This was a regular finding in the decerebrated and spinalized animals but reflex effects could sometimes be obtained in animals which were only decerebrated.

In Fig. 3 is seen the monosynaptic reflex response set up from the nerves to biceps and semitendinosus in a decerebrated and spinalized animal. When rapidly increasing the tension in the medial ligament (indicated by the signal line) the monosynaptic response was facilitated. Fig. 4 illustrates the same reflex effects in the electromyogram from biceps femoris in a decerebrated and spinalized animal. A peculiar effect often seen was the augmentation of the electromyogram at both loading and unloading of the ligament. Furthermore it can be seen that there is a considerable afterdischarge, which was a rather constant finding in the electromyogram from the flexors.

The effects illustrated in Fig. 3 and 4 were easily obtained in the spinalized preparation but were usually absent in the animals which were only decerebrated. Sometimes, however, especially if the animal had a moderate and plastic extensor hypertonus, small reflex effects could be obtained even in the decerebrated preparation. It was often enough to move the contralateral leg gently prior to loading the ligament to get the flexor facilitation in such

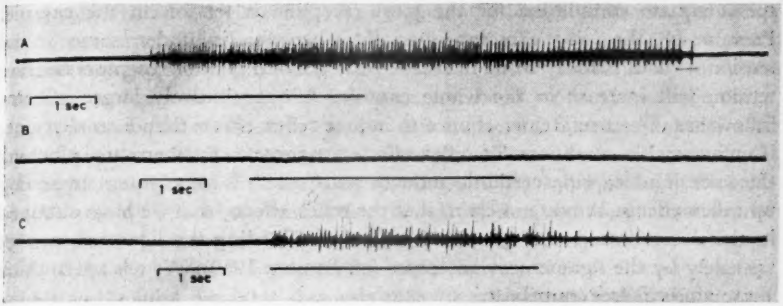

Fig. 5. A. Decerebrated cat. The elctrical activity of the semitendinosus-semimembranosus muscles elicited by loading the medial collateral ligament. The loading was preceded by some manipulation with the contralateral limb. Signal line indicates the time during which the ligament was loaded. B. Same animal. No reflex effects could be elicited after denervation of the contralateral limb. C. Same animal. After spinalization reflex effects reappeared on loading the ligament.

decerebrated animals. Fig. 5 illustrates the reflex effects obtained in a decerebrated animal by loading the ligament in the usual way, indicated by the signal line. In A is seen the electromyographic activity in response to a loading which was preceded by some small manipulations with the contralateral leg. In B the contralateral leg had been denervated and now the effect could not be obtained any longer. In C, on the other hand, the animal had been spinalized and now a reflex effect appeared, though smaller than in A.

Discussion

The experiments presented show beyond doubt that reflex effects are easily set up by stimulation of the knee joint receptors in the cat. The results obtained when stimulating the receptors of the medial collateral ligament do not, however, support the findings of ANDERSSON and STENER (1959).

The difficulty to obtain reflex effects on loading the medial collateral ligament in the decerebrated animal, compared with the ease, with which such effects were obtained in the spinalized preparation, was obvious. We are therefore inclined to think that the failure of ANDERSSON and STENER (1959) to produce any reflex effects was due to the state of excitability in the preparation they used; the decerebrated animal. It might be argued that the difference seen in the decerebrated animal between the effect obtained on pinching or exerting pressure on the joint capsule — which easily produced reflex effects — and the difficulty to induce any on loading the ligament, is significant. The explanation for this difference is probably that the afferent inflow on loading the ligament is much less than on exerting pressure to the capsule. In a recent publication EKLUND and SKOGLUND (1960) showed that

the adequate stimulation for the joint receptors is tension in the capsule. Pressure on the joint capsule, especially when this is under some initial tension — as it is likely to be in situ — will activate a lot of receptors because tension will increase in the whole capsule. A quantitatively larger afferent inflow has of course greater chance to induce reflex effects than a smaller one, if unfavourable conditions for reflex effects are present. Furthermore, pinching the knee joint capsule certainly induces pain which is very potent in setting up reflex effects. We do not claim that the reflex effects, that we have obtained in the decerebrated and spinalized animals on loading the ligament, are set up solely by the ligamentory receptors (cf. STENER 1959). We are apt to think that pain impulses contribute.

It has since long been known that spinalization of decerebrated animals induces marked effects in their spinal reflex pattern (SHERRINGTON and SOWTON 1915, RANSON and HINSEY 1931). In the decerebrated animal the flexors are inhibited, therefore the spinal animal is certainly the preparation of choice for studying flexor reflexes. It might be argued, however, that effects should have appeared in the reflexly active extensors in the decerebrate animal. In this connection we want to draw attention to the types of decerebrate rigidity that can be set up, namely the gamma and the alpha rigidity (GRANIT 1955), which both to some extent contribute to extensor hypertonus in the intercollicular preparation. If, however, the alpha type is prevalent (easily induced by some blood clots under the bony tentorium when decerebrating) the motoneurones might be forced to maximal performance, limiting the fringe available for facilitation, and of course in such states of excitability the extensor motoneurones require strong inhibitory influences to suffer any decrease in their rate of firing. In consistency with the interpretation above, we also found reflex effects most easily elicited in the plastic decerebrated animal with moderate extensor hypertonus (the gamma animal). Another factor that might explain the missing inhibition of extensors in a decerebrated animal is, that in a preparation of the alpha type the gamma mechanism is to some extent inhibited (GRANIT *et al.* 1955). As shown by ELDRED and HAGBARTH (1954) the skin reflexes are working on the gammas in the first hand. A similar mechanism does not seem unlikely for the joint afferents.

In some experiments influences from the contralateral limb were found to be of importance for eliciting reflex effects from the joint. Such influences might be of significance for posture in that contralateral extensor reflexes will preserve the upright position of the animal during ipsilateral flexor reflexes. The appearance of reflex effects in a spinal animal with denervated contralateral limb shows however, the direct influence on the ipsilateral side.

Thus the state of excitability in the spinal cord as well as influences from the contralateral limb were found to be of importance for the elicitation of reflex effects from the knee joint in the cat. That similar excitability states are necessary for the elicitation of joint reflexes in man seems highly probable. The position

of the subject and the electrical activity present in the muscles are known to be of great importance for the production of reflex effects in man (KUGELBERG and HAGBARTH 1958). The failure of PETERSÉN and STENER (1959) to elicite reflex effects from the knee joint in man might be due to unfavourable states of excitability in the motoneuron pools which they tested. They used completely relaxed muscles without any electrical activity present in them.

It appears to us that STENER's interpretation of the hypothesis put forward by PALMER (1958) about the existence of ligamento-muscular protective reflexes, has to be questioned. PALMER clearly states in his paper that an afferent inflow from the joints *during movement* could activate a muscular mechanism protecting the joint etc. This obviously infers something quite different than a protection against violence. We think that violence to the joint by abduction should lead to a flexor reflex, as demonstrated here in the cat, whereby the tension in the collateral ligaments would be decreased to some extent due to the fact that origin and insertion then will be proximated (see BROMAN and HJORTSJÖ 1952). Such a mechanism would, furthermore, fall well in line with the well known general response to noxious stimulation, namely avoidance and withdrawal. The idea of a muscular counteraction to violence especially with the small momentum at hand for the muscles in question also appears very unlikely. We propose that the problem should be reinstated; to what extent do joint receptors contribute to the reflex coordination in normal movements? That problem has not been studied with the methods used, because ligamentory receptors active at flexion and extension are stimulated simultaneously.

Summary

The response of knee flexors and extensors to stimulation of knee joint receptors have been studied in 26 adult cats, decerebrated or decerebrated and spinalized. The limb used was always rigidly fixed and extensively denervated, sometimes the contralateral limb was denervated too. The reflex effects were studied either on the monosynaptic potential set up from various muscle nerves or by electromyography.

1. When stimulating the joint receptors by increasing the intra-articular pressure by means of a syringe filled with Ringer solution, or by squeezing or pinching the anterior part of the capsule, the extensors of the knee were inhibited while the flexors were facilitated. These effects could be produced in the decerebrated and decerebrated and spinalized animal.

2. The receptors of the medial collateral ligament were stimulated by loading a thread sewn around it. This always caused flexor facilitation in the decerebrated and spinalized animal.

3. In decerebrated animals with moderate extensor hypertonus, flexor facilitation could sometimes be obtained when loading the ligament, especially after some preceding manipulation with the contralateral limb.

4. In decerebrated animals it was not possible to get flexor facilitation after denervation of the contralateral limb, or if the animal had a strong decerebrate rigidity.

5. It is not claimed that the afferent inflow from the mechanoreceptors in the joint capsule or the medial collateral ligament are solely responsible for the reflex effects obtained. Pain is very likely to be an important contributing factor.

6. The interpretation of the hypothesis of ligamento-muscular protective reflexes is discussed. It is suggested that the problem, to what an extent joint receptors contribute to reflex coordination in normal movement, should be reinstated.

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ERRATA

EKHOLM, J. and S. SKOGLUND. Intra-articular knee joint temperature variations in response to cooling and heating of the skin in the cat. Acta physiol. scand. 1960. 50. 175—185.

p. 176. line 38: lo *should be* to
p. 176. line 40: 1951 *should be* 1952
p. 177. line 14: symphatectomias *should be* sympathectomies
p. 177. line 41: lumbal *should be* lumbar
p. 177. line 45: sympathectomia *should be* sympathectomy
p. 178. Legend of Fig. 1: muskle *should be* muscle
p. 178. line 3: sympatectomized *should be* sympathectomized
p. 180. Headings of Table I:
i-a-t of intact joint *should be* i-a-t intact side
i-a-t of partly denerv. joint *should be* i-a-t of sympathectomized side
p. 180. line 12: sympathectomia *should be* sympathectomy
p. 181. Headings of Table II:
i-a-t of intact joint *should be* i-a-t of intact side
i-a-t of partly denerv. joint *should be* i-a-t of sympathectomized side
p. 181. Headings of Table III:
i-a-t of intact joint *should be* i-a-t of intact side
i-a-t of partly denerv. joint *should be* i-a-t of sympathectomized side
p. 182. Table IV: No. 20, i-a-t of intact joint, control: 33.05 *should be* 33.00
p. 183. line 13: At *should be* A
p. 185. line 16: proably *should be* probably
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part of the explanation of the paradoxical effects seen, because cooling the intact limb which increases the blood pressure, was not found to produce the phenomenon in the contralateral sympathectomized side. The mechanisms behind the paradoxical phenomena are discussed and it is concluded that temperature variations as measure of bloodflow variations in response to cooling and heating can only be used when they vary in the opposite direction to the stimuli applied.

HORVATH and HOLLANDER (1949) measured intra-articular temperature with thermocouples in the knee joint of man. In a study of the effectiveness of various physical therapy procedures measured as intra-articular temperature variations, they found that application of hot packs on the skin above the joint, which increased the skin temperature, gave a transient decrease of the intra-articular temperature. Cold packs, on the other hand, applied to the skin around the knee, which produced a definite cooling of the skin, increased the intra-articular temperature. HORVATH and HOLLANDER did not give the details of the method used when cooling and heating, neither did they try to find out the mechanism behind these phenomena. They expressed their interpretation of the observation by saying that 'apparently this phenomenon is a sympathetic reflex effect associated with the principle of counterirritation'. They also stated that there is a close correlation between the intra-articular temperature and 'the clinical evaluation of activity of disease'. From their observations they concluded that the temperature of the synovial fluid is directly depending on the relative hyperemia of the synovium.

HUNTER and WHILLANS (1951) measured the intra-articular temperatures in cats, first at room temperature, and then in a low ambient temperature. They found that the intra-articular temperature, on exposure to a low ambient temperature, initially fell below that of the rectal, the muscle and the skin temperatures. The intra-articular temperature initially decreased further when the cat returned to a warm environment. They suggested that this effect was due to a 'reflex superficial vasodilatation resulting in a short period of excessive heat loss'. Such a mechanism was also suggested by UPRUS, GAYLOR and CHARMICHEL (1936), who found a similar paradoxical behaviour of rectal temperature at the beginning of cooling and warming, also observed by GLASER (1949).

COBBOLD and LEWIS (1956) studied the effects of cooling and heating the skin on the blood supply of joints by measuring directly the bloodflow through the knee joint of the dog, instead of measuring intra-articular temperature, which they considered a more indirect method. They flayed one hindlimb, tied off all skin-vessels and replaced the skin which by this procedure is denervated also. Ligatures were put on all branches from the femoral artery except the one to the knee joint. The afferent and efferent leads of a flowmeter were connected to the femoral artery. Rapid cooling by packing chrushed ice around the denervated skin surrounding the external surface of the joint caused a diminution of the flow. When the temperature of the joint was increased, the flow increased which is the normal response to local cooling.

In a plethysmografic study of a knee segment in man, BONNEY, HUGHES and JANUS (1951) found that cooling the segment enclosed in the plethysmograph resulted in a fall of the total bloodflow, while heating gave the reverse effect. When only the superficial circulation was suppressed by adrenalin iontophoresis, the flow was decreased, and additional cooling did not produce

any further decrease. From this experimental result the authors concluded that the articular vessels were relatively unaffected by cooling, and suggested that superficial and deep vessels might have different reactions to variations in temperatures.

The aim of the present experiments was to ascertain if the paradoxical temperature phenomena found in man by HORVATH and HOLLANDER exist in the cat. As indicated by the results of HUNTER and WHILLANS this might be the case at least when the limb is heated. It was thought that such phenomena might be of physiological importance and that the mechanisms underlying them might be the explanation for the great sensitivity to cold observed in arthritic patients. It will here be shown, that such paradoxical phenomena, though small, can be observed in the knee joint of the cat, both after cooling and heating. In order to find out if the mechanisms behind these phenomena includes a nervous link, sympathectomias and denervations were performed.

Methods

Thirty adult cats were used. Some animals were decerebated or decerebrated and spinalized under ether anaesthesia. Some were only curarized, while others were anesthetized with Nembutal 40 mg/kg body weight given i.p.

The temperature recording device was thermocouples. An instrument with great accuracy was used, on which differences of 0.05° C could be measured. Ten different thermocouples could be applied simultaneously, but the temperature recorded by each of them could only be read off one by one with the use of a switchbox.

The intra-articular temperature was recorded by a needle applicator with a diameter of 0.7 mm inserted into the knee joint from the lateral side or through the suprapatellar bursa. Muscle and skin temperatures were recorded in the quadriceps muscle and in the subcutis of the thigh by thermocouples. They were inserted through a syringe-needle which was removed after the thermocouple had been put into the proper position. The rectal temperature was routinely recorded.

The general experimental procedure was to apply a cold pack to an area of about 20 cm^2 on the anterio-medial part of the thigh. This cold pack consisted of a bag filled with crushed ice. The cold pack was removed after a time, that was decided by the response of the intra-articular temperature, which will be further described under Results. When the reactions to cold had been studied, the same area of the thigh was exposed to heating by means of a hot water bottle or a lamp. The experimental observations could be repeated several times in the same animal. In some experiments the animals were exposed to a generalized cooling of long duration, about half an hour, before the effect of heating was studied. The cooling in these cases was accomplished by applying big ice bags around the animal. Sometimes the whole animal was warmed after cooling, and in other cases the whole animal was heated a quarter of an hour before applying the cold pack. All experiments were carried out at a room temperature of 22° C.

In 15 cats a unilateral lumbal sympathectomy was performed. Through a retroperitoneal approach all ganglia from the first lumbar to the ganglion impar were removed on one side. In some of these cats the operation was performed as a sterile one, and these cats were used for experiments one or two months later. These cats are in the following called "chronic". In other cats the sympathectomia was per-

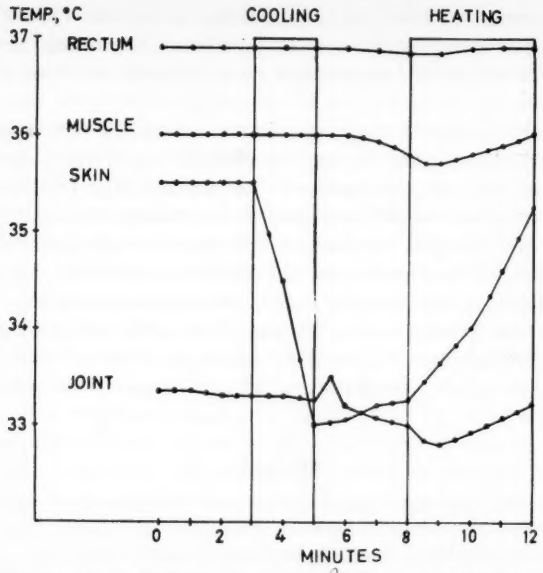

Fig. 1. The temperature variations in rectum, muscle, skin and joint during cooling and rewarming the skin of the anterio-medial aspect of the thigh plotted against time in minutes. For further comments see text.

formed "acute". In all animals a postmortem control was done to ascertain that all ganglia were removed. The experimental procedure used when cooling and heating the sympatectomized animals was generally the same as the one used in intact animals. In one series of experiments, however, the cold pack was applied for a constant time of 2 min.

In another group of animals the same type of experiments with cooling and heating were performed after denervation of the knee joint in one limb. As a rule the medial nerve was cut and in some experiments the posterior nerve in addition. This implies only a partial denervation of the joint because a great many branches reaches it via the muscles (cf. SKOGLUND 1956). The undernervated knee joint served as control.

In still another group of animals the blood pressure was measured in the femoral artery by means of a Hg-manometer during heating and cooling. The animals were heparinized.

Results

Applying a cold pack to the thigh often caused a small rise of the intra-articular temperature, while heating caused a transient increased drop as seen in Fig. 1, which is taken from a typical experiment. It can also be seen that during the procedure the skin temperature fell quickly to a low value and as soon as the cold pack was removed it began to rise.

To obtain a rise of the intra-articular temperature after applying a cold

pack, it was soon found that it was necessary to handle the cold pack in a special way. It had to be applied to the anterio-medial part of the thigh. After cooling for $^1/_2$—2 min it was often observed that the intra-articular temperature began to rise. If at that time the cold pack was immediately withdrawn a small further increase of the intra-articular temperature occurred before it began to fall. In Fig. 1 it can be seen, that after the rise, the intra-articular temperature fell very rapidly, so that already during the first minute after the removal of the cold pack the intra-articular temperature had returned to, or passed below, the control value. After that the intra-articular temperature continued to fall. When, during this later phase, the thigh was quickly rewarmed a new paradoxical reaction of the intra-articular temperature appeared. As seen in Fig. 1 the intra-articular temperature falls even faster than before. After a minute or two, however, it starts to rise. If the cold pack was not removed, when the intra-articular temperature showed a tendency to rise, a very small increase was sometimes seen, but usually none. If the animal, previously to the above described experiments, was exposed to heating of relatively moderate intensity, shown as an increase of about 1° C in the rectal temperature, the paradoxical effects appeared to be of greater magnitude.

The magnitude of the paradoxical response of the intra-articular temperature to cooling varied with the anaesthesia used. The greatest effects, amounting 0.05—0.6° C were seen in curarized preparations. In decerebrated and decerebrated and spinalized animals the increase was about 0.05—0.3° C and still smaller effects were obtained in cats anaesthetized with Nembutal. A steeper fall of the intra-articular temperature constantly occurred on rewarming. Sometimes an additional decrease of up to 0.8° C was seen. The intra-articular temperature responses described were most easily obtained if the tip of the thermocouple was in contact with the synovium of the joint.

If the hindlimb was flayed and the skin sewn on again, neither cooling nor heating could any longer produce a paradoxical response. From this it can immediately be concluded that the paradoxical effects are not a remote effect.

As can be seen in Fig. 1 the muscle temperature was one or two degrees above the joint temperature, this was a constant finding (cf. HUNTER and WHILLANS 1951). The muscle temperature did not show up any paradoxical response to cooling but on the heating of the thigh it showed up such a response.

The skin temperature had quite a different behaviour, which is seen in Fig. 1. It decreases immediately when applying the cold pack, and as soon as the cold pack was removed, it began to rise. The magnitude of the decrease depended on the intensity of the cooling, but the variation was always greater than the one seen in the joint or the muscle. The tip of the thermocouple measuring skin temperature was normally put in the subcutis of the area

Table I. The effect of cooling on the intra-articular temperature (i–a–t) of the intact and sympathectomized sides. By response is meant the highest i–a–t of the intact side within the first minute after removal of the cold pack. The value of the sympathectomized side is taken at the same time. The control is taken just before the cold pack is applied. No. 1—4 are 'acute', 5—6 'chronic' cats all anesthetized with Nembutal. Temp. in °C

No.	Rectal temp. °C	i–a–t of intact joint			i–a–t of partly denerv. joint		
		control	response	difference	control	response	difference
1	36.0	33.00	33.05	+ 0.05	33.10	33.05	− 0.05
2	35.5	31.80	31.90	+ 0.10	32.05	32.00	− 0.05
3	35.8	31.70	31.80	+ 0.10	32.60	32.60	± 0
4	35.0	31.30	31.35	+ 0.05	31.10	31.10	± 0
5	37.0	34.90	34.95	+ 0.05	34.90	34.80	− 0.10
6	36.9	33.55	33.60	+ 0.05	34.90	34.80	− 0.10

where cooling and heating were applied, which explains these great and fast variations.

No decrease of the intra-articular temperature could be observed, when heating was applied initially. If the cat, however, was heated after a preceding cooling of relatively long duration, the muscle and sometimes the rectal temperatures continued to fall with the same, or a lower rate than earlier before it began to rise. The decrease of the joint temperature, on the other hand, was always steeper before it began to rise. The effect on muscle and rectal temperature, which were both observed by GLASER (1949) and HUNTER and WHILLANS (1951), is probably only an indication of that it takes some time before the temperature of the whole animal starts to rise.

Effects of heating and cooling after unilateral lumbar sympathectomy

The reaction of the intra-articular temperature was compared on the intact and sympathectomized sides in both 'acute' and 'chronic' animals. The thermal stimuli were applied simultaneously on the two thighs. A paradoxical rise of the intra-articular temperature to cooling of the sympathectomized side was never seen, but on the intact side the phenomenon was regularly produced. In Table I is seen some values from such experiments. Cooling was performed in the manner described earlier. The highest value within the first minute after removal of the cold pack is given in the table.

After onset of rewarming there is a more marked drop in the intra-articular temperature on the intact side than on the operated one, seen in Table II. The values given, are taken one minute after the onset of heating.

In one series of 'chronic' animals the cold pack was applied on the thigh of the operated side for 2 min while the intra-articular temperature was measured. The value after 2 min was taken. Then the same procedure was performed on the intact side. Considerable attention was devoted to obtain

Table II. The effect of rewarming after cooling on the intra-articular temperature (i–a–t) on the intact and sympathectomized sides. By response is meant the i–a–t 1 min. after onset of the heating. The control is taken just before the heating started. No. 7—9 are 'acute', 10—11 'chronic' cats, all anesthetized with Nembutal. Temp. in °C

No.	Rectal temp. °C	i–a–t of intact joint			i–a–t of partly denerv. joint		
		control	response	difference	control	response	difference
7	35.8	31.80	31.50	− 0.30	32.50	32.50	± 0
8	35.5	31.85	31.70	− 0.15	31.95	32.50	− 0.05
9	35.0	32.10	31.80	− 0.30	31.35	31 35	± 0
10	38.5	36.10	35.70	− 0.40	35.80	35.70	− 0.10
11	37.0	34.00	33.60	− 0.40	34.50	34.40	− 0.10

Table III. The effect of 2 minutes' cooling on the intra-articular temperature (i–a–t) of the intact and sympathectomized sides. By response is meant the i–a–t after the cold pack has been applied for 2 min. exactly. The control is taken just before the onset of the cooling. 'Chronic' cats, curarized only. Temp. in °C

No.	Rectal temp. °C	i–a–t of intact joint			i–a–t of partly denerv. joint		
		control	response	difference	control	response	difference
12	36.7	32.2	32.3	+ 0.1	33.9	33.6	− 0.3
13	35.6	31.9	32.4	+ 0.5	32.9	32.3	− 0.6
14	35.9	33.5	33.3	− 0.2	33.2	32.0	− 1.2
15	36.2	32.8	33.4	+ 0.6	32.9	32.5	− 0.4
16	36.2	33.4	33.2	− 0.2	33.3	32.8	− 0.5

identical experimental conditions on both sides. In Table III the differences between the variations of the intra-articular temperatures of the operated and intact sides are given. On the sympathectomized side a decrease was obtained on cooling while on the intact side an increase or a less marked decrease was seen.

The experimental data from comparisons between the reactions to cooling and heating in the intact and sympathectomized limbs all go to show that an intact sympathetic nervous system is necessary for the appearance of a paradoxical temperature reaction in the joint.

The effects of cooling and heating on partially denervated joints

In one series of experiments the effects of cooling and heating the thigh in the usual manner on the intra-articular temperature of an intact and a partially denervated knee joint were compared (see Methods). Differences in the behaviour of the intra-articular temperature before and after denervation and on the intact and operated sides were looked for, but as can be seen

Table IV. The effect of cooling on the intra-articular temperature (i–a–t) of joints with intact innervation and of partially denervated joints. By response is taken the highest temperature within the first minute after removal of the cold pack. The control is taken just before the cold pack is applied. All cats anesthetized with Nembutal. Temp. in °C

No.	Rectal temp. °C	i–a–t of intact joint			i–a–t of partly denerv. joint		
		control	response	difference	control	response	difference
18	35.2	32.10	32.20	+ 0.10	32.00	32.05	+ 0.05
19	37.5	33.60	33.65	+ 0.05	33.45	33.55	+ 0.10
20	36.0	33.05	33.15	+ 0.15	32.95	33.05	+ 0.10
21	35.8	33.00	33.05	+ 0.05	32.95	33.10	+ 0.15

Table V. The effect of rewarming after cooling on the intra-articular temperature (i–a–t) of joints with intact innervation and of partly denervated joints. By response is meant the i–a–t 1 min. after onset of heating. The control is taken just before heating started. All cats anesthetized with Nembutal. Temp. in °C

No.	Rectal temp. °C	i–a–t of intact joint			i–a–t of partly denerv. joint		
		control	response	difference	control	response	difference
22	35.2	32.05	31.75	− 0.30	31.90	31.55	− 0.35
23	37.5	33.50	33.20	− 0.30	33.20	31.95	− 0.25
24	36.2	32.95	32.70	− 0.25	32.80	32.45	− 0.35
25	35.5	32.60	32.45	− 0.15	32.75	32.55	− 0.20

from the experimental data in Table IV and V the paradoxical phenomena were as readily obtained in the partially denervated knee joint as in the intact.

Blood pressure variations in response to cooling and heating the thigh

In some animals the blood pressure was recorded in the contralateral femoral artery during cooling and heating in the usual way. Cooling was found to give a transient increase of the blood pressure by about 10 % in most animals (from 130—145 mm Hg for instance). Rewarming gave a decrease in the blood pressure which fell even below the control value for half a minute or so. The variations in the blood pressure were found to occur almost simultaneously with the paradoxical temperature variations in the joint. No trials were made to compensate for the variations in blood pressure during cooling and heating to see if the paradoxical temperature effects disappeared. The reason for this was that we considered that the variation in blood pressure can only be part of the mechanism causing the variation in the intra-articular temperature because cooling of the intact side was not found to produce the paradoxical phenomenon in the knee joint of the sympathectomized side.

Discussion

The experiments presented show that the paradoxical phenomena described by HORVATH and HOLLANDER (1949) can be obtained in the knee joint of the cat under certain experimental conditions. From the observation that the paradoxical phenomena could not be produced when the hindlimbs had been flayed it is concluded that there must be some active mechanism and no remote action of the stimulus that influences the intra-articular temperature. It can also be established that the sympathetic outflow is essential for the appearance of the paradoxical phenomenon because it did not appear after lumbar sympathectomy. No definite conclusions can however be drawn from the observation that the paradoxical phenomenon could be produced in joints which had been deprived of a great deal of their innervation. On the other hand it indicates that the nervous mechanism involved is extra-articular.

A lot of works have been done in which the intra-articular temperature has been measured under varying external factors (LONERGAN 1927, HORVATH and HOLLANDER 1949, HUNTER and WHILLANS 1951). HORVATH and HOLLANDER considered the temperature of the synovial fluid to be directly dependent on the estimated hyperemia of the synovium in man. Measurements of the skin temperature have also been done when altering the ambient temperature; the skin temperature has then been used as an indication of vasodilatation and vasoconstriction. That means that a rise of the temperature was interpreted as a vasodilatation and vice versa (LEWIS 1929, UPRUS *et al.* 1936).

The question now arises to what extent temperature variations in the joint can give information about its blood flow. The blood reaching the joint is of higher temperature than the joint itself. Increase of the blood flow must then lead to an increase of the intra-articular temperature if other factors are kept constant. Decrease of flow consequently gives a decrease of the intra-articular temperature under the same conditions. When the ambient temperature is altered, on the other hand, this must also influence the intra-articular temperature directly. The temperature of the blood in the periphery is also altered. If the intra-articular temperature, which is about 31—34° C, increases in spite of a decrease of the ambient temperature, this must mean that either the blood inflow, having a temperature of 35—38° C if its temperature is constant, has increased through the joint and/or that the temperature of the arterial blood actually has increased. If a steeper gradient in the fall of the intra-articular temperature occurs, when the ambient temperature is raised, this must in the same way imply a diminution of the blood flow through the joint. Consequently, when altering the ambient temperature, statement of that the intra-articular temperature is related to blood flow — arterial blood temperature — variations, can only be made when the intra-articular temperature varies in the opposite direction to the applied tem-

perature. This was the case in the experiments presented here. Thus the paradoxical phenomena are probably due to variations in the blood flow, and possibly to variations of arterial blood temperature, at least partly. The total variations in the flow can not be judged from these experiments.

As stated above an intact sympathetic outflow is necessary for the appearance of the phenomenon. Since the sympathetic nerves innervate both skin and joint vessels the paradoxical effects might be explained by two mechanisms: Firstly, on cooling, a contraction of the skin vessels occurs leading to the increase of blood pressure as demonstrated and hence increase of the blood flow directed to deeper parts, even if their vascular lumina are unchanged, secondly a reflex dilatation of joint vessels might occur. The observation that the effect could be produced even if the joint nerves had been cut gainsays that the second mechanism should play any more significant role, but this is not completely proved because the joint was only partly denervated. COBBOLD and LEWIS (1956) who measured the blood flow in the artery to the knee joint found nothing but decrease of it when the hindlimb was cooled. This would contradict our first explanation if in the experiment of COBBOLD and LEWIS the whole limb had not been flayed. This, according to our results, explains why COBBOLD and LEWIS could not confirm the results of HORVATH and HOLLANDER.

It is known that the skin vessels contract on exposure to cold, but it is also known that vasoconstriction is lessened when the skin vessels are deprived of their sympathetic innervation (HERTZMAN 1959). It is also well known that cooling of the skin gives a reflex increase of the blood pressure as found in this investigation. Part of the explanation for the paradoxical phenomenon might therefore be that when the skin is cooled the skin vessels, including the veins contract and force some 'depot' blood towards the heart increasing its output and therefore the blood pressure and probably the flow through the joint vessels. That no paradoxical phenomenon appears in the muscles on cooling might be due to that their vessels are also reflexely constricted which contributes to the increase in the blood pressure. The joint vessels on the other hand might remain unchanged initially (cf. BONNEY *et al.* 1952) allowing an increased flow of blood caused by the increased pressure. That the joint vessels on long cooling will constrict is indicated by the work of COBBOLD and LEWIS (1956). If however the increase in blood pressure was the only cause of the paradoxical phenomenon this should have occurred on the sympathectomized side on cooling the intact, but that was not the case. It must therefore be some local response which contributes to the appearance of the phenomenon. It is possible that the blood flow can be reflexly forced from superficial to deep veins. Normally, the blood will be cooled during the passage in the superficial vessels. On cooling the superficial vessels both arteries and veins contract and the flow will be directed through deeper vessels, including veins, running close to the arteries. According to the prin-

ciple of heat-exchange the blood in the veins will be warmed from the arteries. The above outlined mechanism might be part of the explanation for the paradoxical phenomena. Other possible mechanisms might also be at hand, namely a direction of arterial blood to deeper vessels already at the root of the limb whereby the vein blood exerts less cooling effect on the arterial blood. By such a mechanism the joint would receive arterial blood of higher temperature. This explanation is thus an alternative one, on the line that temperature of the blood and not an increased flow would be the cause of the effects seen.

The explanations proposed above for the paradoxical phenomena are of course only suggestions. However, the measurements of blood pressure variations and the observation of the importance of a local response lend support to the ideas about variations in the blood flow through the joint as the cause of the phenomena seen. Trials to make direct measurements of blood flow through the joint with a drop counter gave very small and inconsistent results probably because the operations necessary caused too much disturbances of the skin vessels.

The effects on the joint temperature observed in the cat were small like those found by HORVATH and HOLLANDER (1949) in man. From that one would be apt to conclude that the paradoxical phenomena seen are part of a physiological mechanism of little importance. Since, however, nothing is known about the total variations in blood flow, such statements would be premature.

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Inhibitory Effects of Hydrochloric Acid in Antrum and Duodenum on Histamine-Stimulated Gastric Secretion in Pavlov and Heidenhain Pouch Dogs

By

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Abstract

ANDERSSON, S. *Inhibitory effects of hydrochloric acid in antrum and duodenum on histamine-stimulated gastric secretion in Pavlov and Heidenhain pouch dogs.* Acta physiol. scand. 1960. *50.* 186—196. — In Pavlov and Heidenhain pouch dogs with excluded antrum and duodenum the effects of HCl instillations into those areas upon histamine-stimulated gastric secretion were studied. It was found that acidification of the duodenum produced slight and irregular inhibition in some dogs, but no inhibition in others. These findings led the author to suggest that histamine-stimulated gastric secretion is resistant to duodenal inhibition. Acidification of the antrum had no inhibitory influence on the secretion. No difference in susceptibility to antro-duodenal inhibition was found between secretions from Pavlov and Heidenhain pouches.

The writer has studied in a series of investigations the inhibitory effects of acid instillation into excluded antrum and duodenum on various types of gastric secretion from vagally innervated and denervated gastric pouches in dogs (ANDERSSON 1960 a, b, c, d). In those investigations an acid reaction in the duodenum had an inhibitory action on all types of secretion studied, the inhibition probably taking place at parietal cell level and being independent of the vagal innervation to the pouch; furthermore, an acid reaction in the antrum had a conspicuous inhibitory effect only upon secretion elicited by the vagal route (insulin hypoglycemia).

Histamine, a potent stimulus of the HCl-producing gastric glands, is commonly used in studying the influence of inhibitors on gastric secretion. Previous

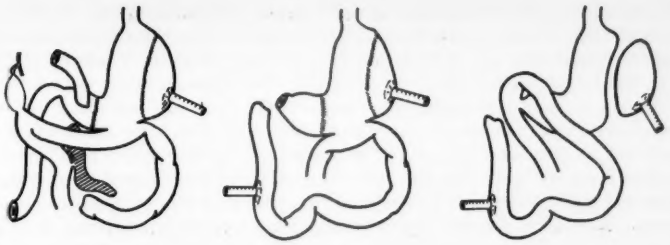


Fig. 1. Operative preparation of the dogs:
I. (Dog 115) Pavlov pouch; mucosal wall between the corpus and antrum; gastrojejunostomy; cholecystojejunostomy and transplantation of the main pancreatic duct to the jejunum; antral and duodenal fistulas. (Dogs 111 and 112) Heidenhain pouches; preparation otherwise as in dog 115.
II. (Dogs 75 and 137) Pavlov pouch; mucosal wall between the corpus and antrum; gastrojejunostomy; antral fistula and cannula in the duodenum.
III. (Dogs 136 and 143) Heidenhain pouch; pylorojejunostomy and cannula in the duodenum.

investigations into the responses of histamine-induced secretion to antro-duodenal inhibition have given inconsistent results. Some authors (WOODWARD et al. 1954) have reported that histamine-stimulated secretion is not inhibited by acidification of the *antrum;* others (JORDAN and SAND 1957, WOODWARD et al. 1958) have obtained contradictory results. Similar inconsistencies are found with regard to the effect of acid in the *duodenum* on the above-mentioned type of secretion. Thus PINCUS et al. (1944) were unable to influence histamine-stimulated secretion from Pavlov pouches by acid instillation into the duodenum, whereas CODE and WATKINSON (1955) and SIRCUS (1958) reduced the secretion in that way.

It was recently shown (ANDERSSON 1960 d) that secretion stimulated by intravenous administration of gastrin was depressed by intra-duodenal instillation of HCl. This observation was considered to indicate strongly that duodenal inhibition occurred at parietal cell level. In the preceding investigation the action of antro-duodenal instillations of acid on histamine-stimulated gastric secretion was studied.

Method

Seven adult mongrel dogs weighing 15—20 kg were used. Pavlov pouches were established in three of them (no. 75, 115 and 137) and Heidenhain pouches in the other four (no. 111, 112, 136 and 143). Three different modes of preparation were subsequently employed.

Group I (no. 111, 112 and 115): When the inhibitory effects of acid instillations into excluded antrum-duodenum region were studied in earlier investigations (ANDERSSON 1960 a, c), the acid was instilled via a cannula in the antrum. No reliable determinations of the antro-duodenal pH were practicable in these experiments. To facilitate the maintenance of a constant, low pH in the antrum-duodenum and in order to keep it under

satisfactory control, the following mode of operative preparation was devised. About three weeks after the gastric pouches had been established, antrum-duodenum exclusion and gastrojejunostomy were performed by a method reported elsewhere (ANDERSSON 1960 b). When the animals had recovered from this operation they were subjected to the following surgical measures. The common bile duct was mobilized and divided between ligatures, as was the accessory pancreatic duct. The main pancreatic duct was exposed and its distal end cut out of the duodenum together with a small piece of the contiguous duodenal wall. The bile flow to the intestine was restored by an anastomosis between the gallbladder and the jejunal loop running from the gastrojejunostomy. Approximately 15 cm distal to the anastomosis the mobilized pancreatic duct was implanted. The duodenum was divided at the ligament of Treitz; the distal end was closed and the proximal end brought out to the surface to form a cutaneous fistula. A fistula to the antrum was established by means of a resected part of the terminal ileum. It facilitated the perfusions of the antrum-duodenum sector and eliminated the possibility of rises in intra-antral pressure during them. Details of the preparation of these animals will be found in Fig. 1 : I.

Group II (*no. 75 and 137*): These dogs too were subjected to antrum-duodenum exclusion as well as gastrojejunostomy. At a third operation the pylorus was divided. The duodenal end was closed and the antral end brought out through the abdominal wall as a cutaneous fistula. A cannula was inserted into the duodenum about 10 cm distal to its upper end (Fig. 1: II).

Group III (*no. 136 and 143*): When the animals had recovered from the first operation they underwent the following surgery: The pylorus was divided and the duodenum closed. A new gastrointestinal passage was established by anastomosing the first loop of the jejunum to the divided pylorus. A duodenal cannula was inserted. The preparation is illustrated in Fig. 1 : III.

Before initiation of the experiments the animals were allowed at least three weeks to recover. For further details of operative methods and postoperative management, the reader should refer to ANDERSSON, ELWIN and UVNÄS (1958) and ANDERSSON (1960 b).

Each experiment was started in the forenoon after the animals had fasted for 18—24 hours. The basal secretion was recorded for at least one hour before the start of stimulation of secretion with histamine. Histamine dihydrochloride (the histamine doses recorded in the tables and figures are expressed in the amount of histamine dihydrochloride) was dissolved in sterile physiological saline in concentrations of 20—50 μg per ml. With the aid of an automatic infusion pump the solution was continuously administered intravenously over 3—5 hours, a constant secretory level generally being reached after 1—2 hours. The doses infused were so adjusted as to produce a secretory response of less than 50 per cent of the maximum secretory output of the pouch. Any given animal received a uniform dose of histamine in each experiment, *i. e.*, the dose was not correlated to the animal's weight. In the tables and figures, however, the doses are recorded in μg per kg. Minor variations in an animal's weight from one experiment to another therefore gave rise to differences in the amounts of histamine administered per kilogram. The differences in doses were nevertheless small, and such variations in secretory responses as were observed could not be attributed to them, since a somewhat larger dose of histamine often produced a lesser secretory response than did a smaller dose (cf. the experiments in Table II A, IV and V).

The gastric secretory output was collected in 15-minute portions and the amounts of free HCl and total acid were determined by titration of each portion against N/100 NaOH, with Töpfer's reagent and phenolphthalein as indicators. The term "secretory rate", used in Fig. 2—4, represents the milliequivalents of total acid secreted per 15 minutes.

Table I. Secretory responses to histamine in Pavlov pouch dog with and without concomitant instillation of HCl into the antrum-duodenum (Dog 115)

	Exp. no.	Histamine dose µg/kg/min	Secretion (mEq total acid)						
			Control period (one hour)	Response to histamine					
				1st hr	2nd hr		3rd hr	4th hr	
					0.5 hr	0.5 hr		0.5 hr	0.5 hr
Controls	1	0.29	0.04	0.98	0.74	0.77	1.56	0.72	—
	2	0.29	0	0.82	0.95	0.91	1.58	0.55	—
	3	0.29	0.02	0.68	0.57	0.57	1.02	0.56	—
	Mean		0.02	0.83	0.75	0.75	1.39	0.61	—
N/10 HCl in antrum-duodenum during half of the 2nd, the 3rd, and half of the 4th hours	1	0.29	0.05	0.62	0.56	**0.87**	**1.53**	**0.93**	—
	2	0.29	0.03	0.83	0.54	**0.95**	**1.75**	**0.70**	—
	Mean		0.04	0.73	0.55	**0.91**	**1.64**	**0.82**	—
In percent of mean for controls			—	88	73	**121**	**118**	**134**	—

The effects of HCl infusions into the antrum and duodenum upon the secretory responses to histamine were studied both during the initial phase of the responses and during the later stages when the level was relatively constant. The acid infusions proceeded for 1—2 hours. Perfusions of the isolated antrum-duodenum in dogs 111, 112 and 115 were effected as follows. The acid was instilled through a rubber catheter inserted into the antrum via the antral fistula. To facilitate passage of the instilled solution into the duodenum, another rubber catheter (perforated) was advanced via the duodenal fistula, into the antrum. For acid perfusions of the antrum-duodenum in these dogs, N/10 HCl was used. The duodenum in these animals was devoid of the neutralizing action of bile and pancreatic juice; at the instillation rates used, *i. e.*, about 100 ml per hour, the pH of the instilled acid solutions thus rose only very slightly. After perfusion the pH of the acid solutions was at no time higher than 1.5. Acid of the same strength was used for perfusion of the antrum in dog 137. At a perfusion rate of approximately 100 ml per hour the intra-antral pH varied between 1.2 and 1.5. For reasons mentioned in a previous paper (ANDERSSON 1960 b), N/5 HCl was used for duodenal instillations in dogs 75, 136, 137 and 143. Infusion of approximately 100 ml acid per hour reduced the intra-duodenal pH to about 2. For further details of the perfusion technique used in the last-mentioned animals, the reader is referred to ANDERSSON (1960 b).

Results

A. *Instillation of Acid into the Antrum-Duodenum*

In nine experiments on one Pavlov pouch dog and two Heidenhain pouch dogs prepared according to Fig. 1 : I, the secretory responses to continuous intravenous infusion of histamine were determined. In 8 experiments the influence of acid instillations into the excluded antrum-duodenum upon those

Table II. Secretory response to histamine in Heidenhain pouch dogs with and without concomitant instillation of HCl into the antrum-duodenum

A. Dog 111.

	Exp. no.	Histamine dose μg/kg/min	Secretion (mEq total acid)				
			Control period (one hour)	Response to histamine			
				1st hr	2nd hr		3rd hr
					0.5 hr	0.5 hr	
Controls	1	0.18	0.37	1.39	0.78	0.88	2.31
	2	0.20	0.06	0.61	0.57	0.70	1.47
	3	0.20	0	0.51	0.71	0.82	1.79
	Mean		0.14	0.84	0.69	0.80	1.86
N/10 HCl in antrum-duodenum during half of the 2nd and the 3rd hours	1	0.20	0.21	1.16	1.00	**0.92**	**1.83**
	2	0.18	0.01	1.00	0.83	**0.88**	**1.20**
	3	0.18	0	0.52	1.15	**1.29**	**2.43**
	Mean		0.07	0.89	0.99	**1.03**	**1.82**
In percent of mean for controls				106	143	**129**	**98**

Table II

B. Dog 112.

	Exp. no.	Histamine dose μg/kg/min	Secretion (mEq total acid)				
			Control period (one hour)	Response to histamine			
				1st hr	2nd hr	3rd hr	4th hr
							0.5 hr	0.5 hr
Controls	1	0.27	0	0.58	1.53	1.73	1.06	—
	2	0.29	0	0.47	0.92	1.18	0.68	—
	3	0.25	0	0.29	0.73	0.95	0.46	—
	Mean		0	0.45	1.06	1.29	0.73	—
N/10 HCl in antrum-duodenum during the 3rd and half of the 4th hour	1	0.27	0	0.46	1.02	**1.42**	**0.72**	—
	2	0.27	0	0.25	0.87	**1.41**	**0.72**	—
	3	0.27	0	0.42	1.14	**1.43**	**0.50**	—
	Mean		0	0.38	1.01	**1.42**	**0.65**	—
In percent of mean for controls				84	95	**110**	**89**	

Table III. Secretory responses to test meal in Pavlov pouch dog with and without concomitant instillation of HCl into the antrum-duodenum (Dog 115. Test meal: 25 g meat)

	Exp. no.	Secretion (mEq total acid)				
		Control period (one hour)	After test meal			
			1st hr	2nd hr	3rd hr	4th hr
Controls	1	0.03	2.31	1.41	0.83	1.16
	2	0	1.96	2.19	1.86	0.89
	3	0.04	2.65	1.43	0.80	0.28
	4	0.01	1.99	1.69	0.88	0.55
	5	0.04	2.68	1.64	1.17	0.40
	Mean	0.02	2.32	1.67	1.11	0.66
N/10 HCl in antrum-duodenum during the 1st hour	1	0.04	**0.37**	1.74	1.44	0.50
	2	0.13	**0.51**	1.21	1.00	0.62
	3	0.02	**0.80**	0.46	1.27	0.66
	4	0.01	**0.53**	1.00	0.87	0.44
	5	0.06	**0.82**	0.92	0.50	0.17
	Mean	0.05	**0.61**	1.07	1.02	0.48
In percent of mean for controls		—	**26**	64	92	74

responses was studied. The acid infusions in these experiments were not initiated until the secretion had reached a constant level. The results are presented in Table I, II A and B. The findings indicated that acid in the antrum-duodenum had no inhibitory effect upon histamine-stimulated secretion either from Pavlov or Heidenhain pouches.

Secretory responses to test meals, on the other hand, were markedly inhibited by acid perfusion of the isolated antrum-duodenum (see Table III).

B. *Instillation of Acid into the Antrum*

In one of the Pavlov pouch dogs (no. 137) with isolated antral pouch, the effect of antral acidification upon histamine-stimulated secretion was studied in three experiments. The results are shown in Fig. 2. The mean secretory output per 15 min during the hour immediately preceding the acid infusion is taken as 100 per cent (control level). The secretion is expressed in per cent of the control level. No inhibition was observed.

C. *Instillation of Acid into the Duodenum*

Experiments of two types were conducted: In series a) acid instillation and stimulation of the secretion were initiated concurrently; in series b) the acid infusion was not started until the secretion had reached a constant level.

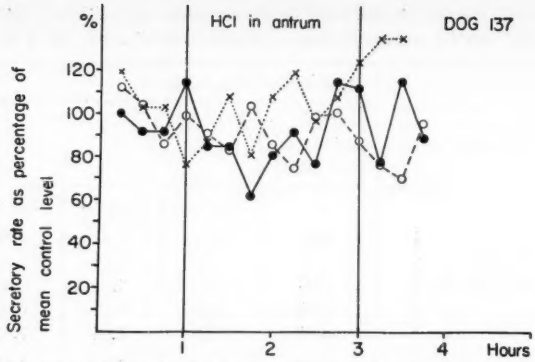


Fig. 2. Attempt to inhibit histamine-induced gastric secretion from a Pavlov pouch by HCl perfusion of antrum (intra-antral pH 1.2—1.5). Each curve represents a separate experiment. The 100 per cent level is the mean 15-minute gastric secretion (control level) during the hour immediately preceding the infusion of acid. Histamine dose 0.43—0.44 μg/kg/min. Mean secretory control level varied between 0.19 and 0.39 mEq acid per 15 min.

Series a) (Dogs 75 and 143)

In one Pavlov and one Heidenhain pouch dog the secretory responses to histamine were determined in 11 experiments. In 7 other experiments the influence of a low intra-duodenal pH on those responses was studied. Acid

Table IV. Secretory responses to histamine in Pavlov pouch dog with and without concomitant instillation of HCl into the duodenum (Dog 75)

	Exp. no.	Histamine dose μg/kg/min	Secretion (mEq total acid)			
			Control period (one hour)	Response to histamine		
				1st hr	2nd hr	3rd hr
Controls	1	0.36	0.21	1.14	1.26	0.84
	2	0.36	0.32	1.73	4.13	—
	3	0.34	0.33	2.77	2.84	3.76
	4	0.34	0.07	1.50	2.55	3.62
	5	0.32	0	1.37	1.94	3.11
	6	0.32	0.14	1.50	2.53	3.70
	Mean		0.18	1.67	2.54	3.01
N/5 HCl in duodenum during the 1st and 2nd hours	1	0.33	0.26	**1.20**	**2.24**	1.49
	2	0.36	0.12	**1.44**	**1.85**	2.65
	3	0.32	0.09	**1.50**	**2.86**	2.64
	4	0.34	0.32	**1.82**	**2.49**	2.39
	Mean		0.20	**1.49**	**2.36**	2.29
In percent of mean for controls				**89**	**93**	76

Table V. Secretory responses to histamine in Heidenhain pouch dog with and without concomitant instillation of HCl into the duodenum (Dog 143)

	Exp. no.	Histamine dose µg/kg/min	Secretion (mEq total acid)					
			Control period (one hour)	Response to histamine				
				1st hr	2nd hr		3rd hr	
					0.5 hr	0.5 hr	0.5 hr	0.5 hr
Controls	1	0.20	0.07	0.57	0.47	0.60	0.65	—
	2	0.18	0	0.99	0.63	0.68	0.74	—
	3	0.17	0.30	0.77	0.46	0.53	0.56	—
	4	0.17	0.15	0.58	0.27	0.34	0.36	—
	5	0.17	0.29	0.56	0.42	0.48	—	—
	Mean		0.16	0.69	0.45	0.53	0.58	—
N/5 HCl in duodenum	1	0.19	0.04	**0.56**	**0.59**	0.55	0.55	—
during the 1st and half	2	0.18	0.05	**0.35**	**0.68**	0.65	0.62	—
of the 2nd hour	3	0.18	0	**0.40**	**0.61**	0.82	0.77	—
	Mean		0.03	**0.44**	**0.63**	0.67	0.65	—
In percent of mean for controls				**64**	**140**	126	112	—

instillation, as shown in Table IV and V, had no demonstrable effect on the secretion in the Pavlov pouch dog; the secretory response with concomitant acid instillation averaged approximately 90 per cent of that in the controls. In the Heidenhain pouch dog the acid instillation caused some initial depression of the secretion (64 per cent of the control level during the first hour), but this effect subsided during the next half hour of the instillation period, when the secretion rose to 140 per cent of that in the controls.

Series b) (*Dogs 136 and 137*)

In one Pavlov and one Heidenhain pouch dog the acid instillation was not begun until the secretion had reached a relatively constant level. Fig. 3 A and B show the effects of duodenal instillations of HCl in these two dogs. As in Fig. 2 the secretion is expressed in per cent of mean control level, *i. e.* the mean secretory output per 15 min during the hour immediately preceding the acid infusion. Some inhibition of the secretion during the instillation period can be observed in each of them. In the Pavlov pouch dog, however, the inhibition was not significant in all experiments. In no instance did the secretion fall below 60 per cent of the control level. In the Heidenhain pouch dog it frequently failed to return to the control level when the instillation ceased.

The results did not differ in principle when the secretion was expressed in milliequivalents of free HCl instead of milliequivalents of total acid.

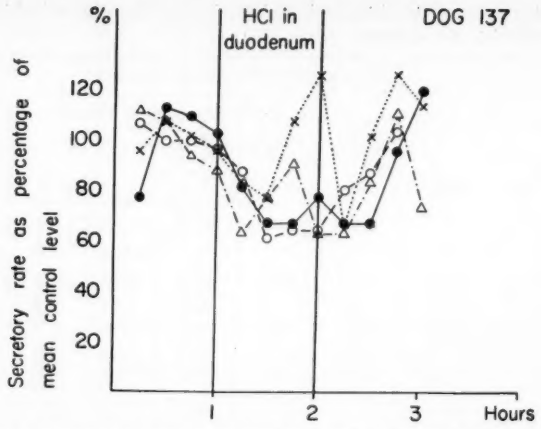
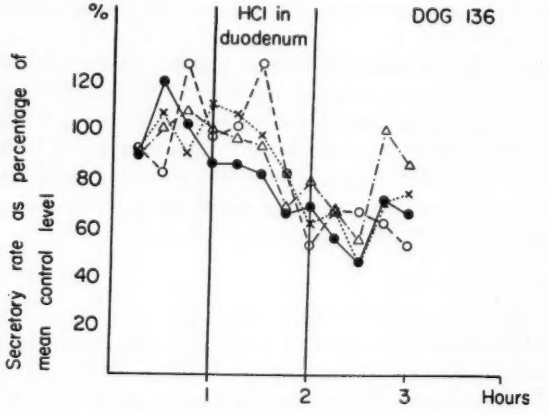


Fig. 3. Inhibition of histamine-induced gastric secretion by instillation of HCl into the duodenum (intra-duodenal pH about 2). Each curve represents a separate experiment. The 100 per cent level is the mean 15-minute gastric secretion (control level) during the hour immediately preceding the infusion of acid.

A. Pavlov pouch dog. Histamine dose 0.42—0.44 μg/kg/min. Mean secretory control level varied between 0.17 and 0.31 mEq acid per 15 min.



B. Heidenhain pouch dog. Histamine dose 0.24—0.26 μg/kg/min. Mean secretory control level varied between 0.21 and 0.30 mEq acid per 15 min.

Discussion

The experiments demonstrate that histamine-stimulated secretion, in contrast to other types of secretion studied (ANDERSSON 1960 a, b, c, d), is markedly resistant to inhibitory influences from the antrum-duodenum region; for in-

stillation of HCl into the antrum and duodenum had no inhibitory effect in 5 dogs, while instillation into the duodenum alone produced minor inhibition in 2 animals. It is not surprising, therefore, to find that previous investigations into the effect of HCl instillation into the antrum and duodenum on histamine-stimulated secretion have yielded divergent results.

Influence of Low Intra-Duodenal pH upon Histamine-Induced Gastric Secretion

PINCUS et al. (1944) were unable to depress histamine-stimulated secretion from Pavlov pouches if the intra-duodenal pH was reduced to 2. They did, however, inhibit other types of secretion (stimulated by test meals or hypoglycemia). CODE and WATKINSON (1955) as well as SIRCUS (1958) have since asserted that histamine-induced secretion from Pavlov pouches is inhibited by instillation of HCl into the duodenum. CODE and WATKINSON found no significant inhibition if the secretion exceeded 60 per cent of the maximum secretory response of the pouch to histamine. The same authors, on the other hand, recorded a marked inhibition of the secretory responses to test meals even though most of those responses exceeded the maximum response of the pouch to histamine.

The present results show the same inconsistencies as those in the above-mentioned investigations. In five animals (two Pavlov and three Heidenhain pouch dogs) acidification of the duodenum did not affect the secretion. In two (one with a Pavlov and one with a Heidenhain pouch) there was a minor inhibition of the secretion.

Under certain conditions it seems possible, therefore, in some dogs to inhibit histamine-stimulated secretion by acidification of the duodenum. However, the depression is relatively slight and difficult to produce; thus it contrasts sharply with the regular and very pronounced inhibition that has been recorded in other types of secretion investigated (ANDERSSON 1960 a, b, c, d).

The effects observed here of intra-duodenal instillation of HCl on histamine-stimulated secretion are neither unequivocal nor sufficiently assured to permit further discussion on the nature of the duodenal inhibition.

Influence of Low Intra-Antral pH Upon Histamine-Induced Gastric Secretion

Several authors have suggested in recent years that a secretion inhibiting factor is liberated from the antrum at a low intra-antral pH. Support for this hypothesis was presented by *e. g.* JORDAN and SAND (1957), who reported that histamine-stimulated secretion from Heidenhain pouches was inhibited by perfusion of an isolated antral pouch with hydrochloric acid. The inhibition did not occur until perfusion had proceeded for 1—3 hours. Their results were subsequently duplicated by WOODWARD et al. (1958), who, however, brought up the possibility that inhibition occurring after such a long latent period might be due to a toxic effect of the prolonged infusion. WOODWARD et al. (1954) had previously disputed the view that histamine-stimulated secretion could be inhibited by acidification of the antrum.

In three dogs used in the present investigation (one with a Pavlov and two with Heidenhain pouches) the isolated antrum-duodenum was perfused with acid for $1^1/_2$—2 hours. The intra-antral pH in these animals was low (1—1.5), yet no inhibitory effect could be detected. In one Pavlov pouch dog with isolated antral pouch, acid perfusion of the antrum for 2 hours had no effect on the secretory responses. Even though the latent period for antral inhibition is between one and three hours, it seems reasonable to assume that some trace of inhibition would have been detectable even if the perfusions in these experiments had not exceeded $1^1/_2$—2 hours duration. These results do not corroborate the claim that a hypothetical antral secretion-inhibiting factor is able to influence histamine-stimulated secretion.

Histamine cannot, therefore, be regarded as a suitable stimulus for studying the activity of secretion-inhibiting mechanisms localized in the region of the antrum and duodenum, and it would seem impracticable, moreover, to draw any conclusions concerning the nature of such mechanisms from studies of gastric secretion in which histamine is used as a stimulus.

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